Vol. LXXV, No. 2 October, 1938

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE ACTIVITIES OF VARIOUS SUBSTITUTED PHENOLS IN STIMULATING THE RESPIRATION OF SEA URCHIN EGGS[1]

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INTRODUCTION

Whenever a series of related compounds is found to be capable of producing a given physiological effect, there always appears the possibility of relating the activities of the various substances to their molecular structure. In examining such possible relations it is important to distinguish between two different measures of the activity of a substance. Where the effect increases continuously with increasing concentration of the substance, the activities may be represented by the concentration required to produce an effect of a definite magnitude. But if the magnitude of the effect passes through a maximum with increasing concentration of the agent, the activity may be defined by the concentration required to produce the optimum (or maximum) effect or it may be defined by the magnitude of the optimum effect. It is also essential, when more than one molecular species of a substance exists, to determine which is the active form. Without such information no real comparison of activities can be made, since with different substances the concentration of the active form will depend upon the equilibrium conditions existing between the various molecular species.

Due principally to the work of Clowes and Krahl (1936) a large number of substituted nitro- and halophenols are now known that are capable of increasing the respiratory rate of marine eggs. They also exhibit the highly interesting reversible block to cleavage first demonstrated by these investigators (1934) with 4,6 dinitro-o-cresol. The effect of these agents is of further interest in that the increased respiratory rate is not accompanied by abnormal development, as is the case with the various oxidation-reduction dyes that had been previously (Barron, 1929; Runnström, 1930) shown to stimulate the

[1] This investigation was supported in part by a grant from the Penrose fund of the American Philosophical Society.

respiration of marine eggs. These substances are then of interest to the embryologist since there exists the possibility of harnessing the extra energy that is liberated, and the ability to stop cleavage in a reversible manner should help us discover the factors that cause it to go.

The substituted phenols are all weak monobasic acids and the question of the active molecular species relates to whether it is the anion or the undissociated molecule that is effective. In a previous publication (1937) we have presented evidence for the view that it is the anion that is the active form inside the cell, but that penetration must be effected as the undissociated molecule. The evidence is based in part on the existence of a pH effect. At lower pH's it requires a lower concentration of a given substituted phenol to produce the maximum respiratory stimulation or reversible block to cleavage than it does at higher pH values of the solution. But the calculated concentration of undissociated molecules is very closely the same at the different pH's. Field et al. (1934) first demonstrated this pH effect of 2,4 dinitrophenol on yeast. He interpreted the result to mean that the substance is active as the undissociated molecule. However, when we compared a number of different nitro- and halophenols on the basis of the concentration of undissociated molecules present at maximum effective total concentration, enormous differences were found. It appeared for the extremes that a million molecules of one substance would be required to produce the same effect as one molecule of another if the undissociated molecule were the active form. On the other hand, when the concentration of the anion inside the cell was estimated, assuming that the substances penetrate freely in the undissociated form, and using the value for the internal pH determined by Chambers and Pollack (1927) and the Needhams (1926) a more reasonable result was obtained. The different compounds then gave values of the same order of magnitude.

The calculations of the concentrations of the undissociated molecules and of ions in the solution and inside the cell are made from the usual mass action equations. The concentration of undissociated molecules, $[HP]$, present in the solution may be expressed as a function of the hydrogen ion concentration, $[H^+]$, and the total concentration, $[\Sigma P]$, by the equation:

$$[HP] = \frac{[H^+][\Sigma P]}{[H^+] + K}, \tag{1}$$

where K is the constant for the acid dissociation of the particular substituted phenol and $[\Sigma P] = [HP] + [P^-]$. The concentration of anions, $[P^-_i]$, inside the cell is given simply by the equation:

$$[P^-_i] = \frac{[HP_i]\,K}{[H^+_i]} \tag{2}$$

in which the subscript, i, refers to values for the inside of the cell. There are several assumptions involved here. Firstly, the value of $[HP_i]$ is taken to be the same as $[HP]$ in the solution. This follows from the evidence that the substituted phenols penetrate freely in the undissociated form. Secondly, it is assumed that activity coefficients may be neglected. The error involved in this assumption is certainly very small in comparison with other sources of error in the data. It is assumed, thirdly, that $[H^+_i]$ is not changed by the addition of these substituted phenols. We shall discuss this point below.

Combining equations (1) and (2) we have:

$$[P^-_i] = \frac{[H^+_o][\Sigma P]\,K}{[H^+_i]([H^+_o] + K)}, \tag{3}$$

where the subscript, o, refers to the solution and the subscript, i, to the inside of the cell. In the cases where K is large with respect to $[H^+_o]$ then $[P^-_i]$ will vary directly with the hydrogen ion concentration of the solution and with total concentration of substituted phenol, namely:

$$[P^-_i] = \frac{[H^+_o][\Sigma P]}{[H^+_i]}. \tag{4}$$

Where K is small with respect to $[H^+_o]$ we have

$$[P^-_i] = \frac{[\Sigma P]\,K}{[H^+_i]}. \tag{5}$$

In the situation represented by equation (4), $[P^-_i]$ is independent of K. Therefore those compounds having relatively large values of K will give the same value of $[P^-_i]$ when $[\Sigma P]$ and $[H^+_o]$ are the same. $[HP]$ at the same time will vary inversely with K as may be seen from equation (1). In the situation represented by equation (5), $[P^-_i]$ is independent of $[H^+_o]$. Therefore those compounds with small values of K will show a direct proportionality between $[P^-_i]$ and K for the same $[\Sigma P]$. $[HP]$ at the same time will be practically equal to $[\Sigma P]$. In either situation, then, $[P^-]$ and $[HP]$ will vary in a different manner, so it will not matter whether we investigate substances with large or with small dissociation constants, provided that the different compounds have sufficiently different dissociation constants. In other words, we could not hope to determine the active form if all the members of the series had very closely the same dissociation constant.

If the dissociation constants for the different substituted phenols were all small or all large we would want to know their values very exactly. But since in our series there are compounds with large values as well as small values available, this is not so essential.

The experiments reported here are an extension of previous work (1937) and involve a comparison of the activities of various substituted nitro- and halophenols. We employ here the first criterion of activity referred to above; namely, the concentration required to produce a definite effect. Data on the magnitude of the maximum stimulation are also presented. In addition a new type of experiment is reported in which the internal pH is altered, and the effect on the optimum concentration determined. Also the buffer capacity of eggs is estimated.

Material and Methods

The eggs of the sea urchins, *Strongylocentrotus purpuratus* (at Corona del Mar, California) and *Arbacia punctulata* (at Woods Hole, Massachusetts), were used in these experiments. These two forms behaved very much alike with respect to the concentrations of the substituted phenols required for maximum respiratory stimulation and reversible cleavage block as well as the magnitude of stimulation.

The oxygen consumption measurements were made by means of the Barcroft-Warburg manometers using the conical type of vessel with side-arm (Anhang). The substituted phenols are added from the side-arm after a preliminary series of readings (lasting usually one hour) on the untreated eggs. The solutions were buffered by means of glycylglycine, which we have recently (1937) shown to be highly suitable for buffering in the neighborhood of pH 8.0. The concentration of glycylglycine was generally 0.015 molar in carbonate-free sea water, prepared as previously described. The pH's were determined by means of the glass electrode (Beckman pH meter), the values for the egg suspensions in the vessels at the end of a run being taken as well as that of the initial solutions.

Results

The nitro- and halophenols[2] listed in Table I were investigated. In all cases a series of at least six different concentrations were employed in order to determine the one giving maximum stimulation. From the theoretical considerations based on the assumption that the

[2] The dichlorophenols were generously supplied by Dr. John Ruhoff of the Mallinckrodt Chemical Works, and were subsequently purified and their melting points checked. The other compounds were obtained from the Eastman Kodak Company.

substances are active as the anion, $[P^-_i]$ inside the cell, it was possible to determine what total concentration to employ at a given pH of solution. Thus a fairly closely graded series of concentrations could be used, which, if the assumptions were correct, should include that at which maximum respiratory stimulation is obtained.

The criterion for maximum respiratory stimulation involves a point which has not been emphasized in previous work with substituted phenols. With increasing concentration the respiratory rate

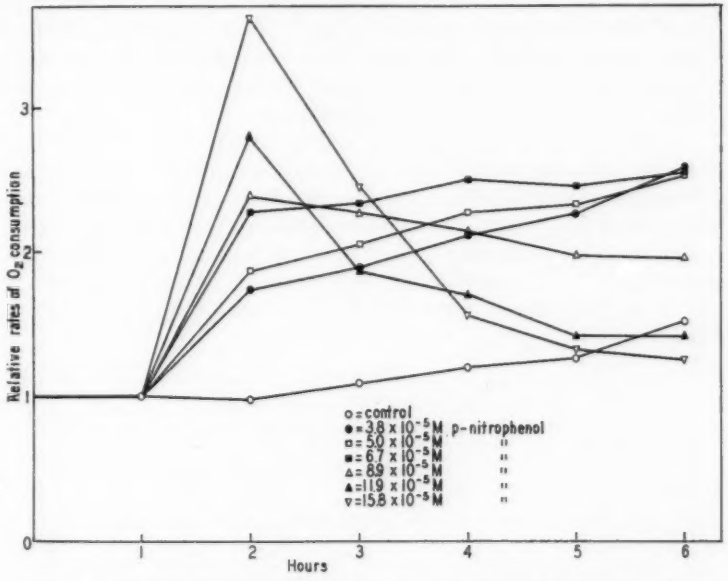

FIG. 1. Rates of oxygen consumption of eggs of *Strongylocentrotus purpuratus* in various concentrations of p-nitrophenol in glycylglycine-buffered sea water at pH 7.90. The reagent is tipped into the egg suspension at the end of the first hour. The relative rates of oxygen consumption are given on the basis of the oxygen consumption during the first hour as unity. Measurements begun at 43 minutes after fertilization. Temperature = 20° C.

increases, but beyond a certain concentration the increase does not remain constant with time. There is, at these higher concentrations, an initial rise in respiratory rate lasting for a short period of time followed by a steady drop in rate, which may finally reach values below that of the controls. This can be seen in Fig. 1, in which the rates of oxygen consumption in different concentrations of p-nitrophenol are plotted against time. With concentrations up to 6.7×10^{-5} molar the rate rises with time as does the control (untreated eggs) rate during

this interval. But above this concentration there is an initial increase in rate followed by a steady drop, which in the case of the two highest concentrations, falls at the end of 6 hours to a value below that of the controls. This kind of variation of the oxygen consumption rates with time is found with the higher concentrations of all the substituted phenols that we have investigated. It is evident, with concentrations giving this initial rise followed by a decrease, that the particular value taken to represent the rate will depend upon the length of time after addition of the reagent during which the respiration is followed. One cannot, then, very well specify the amount of stimulation at these higher concentrations. We shall therefore take as the optimum concentration that concentration giving the greatest increase in rate of respiration without a subsequent decrease.

At the optimum concentration defined in this way we find that cleavage proceeds normally. The reversible cleavage block appears at concentrations just above this optimum; i.e., at concentrations at which there is an initial stimulation followed by a decrease. Thus in the experiment illustrated in Fig. 1, cleavage was blocked in the three highest concentrations of p-nitrophenol. Examination of the eggs from the vessels one hour after the end of the run gave the following percentages in the various stages:

	Number of Cells					Early blastulae
	1	2	4	8	16	
Control	1	1	0.5	0.5	0.5	96.5
3.8×10^{-5} M	1	1	0.5	0.5	0.5	96.5
5.0×10^{-5} M	1	1	0.5	0.5	0.5	96.5
6.7×10^{-5} M	1	1	0.5	0.5	0.5	96.5
8.9×10^{-5} M	1	1	0.5	30	64.5	3
11.9×10^{-5} M	1	39	60	0	0	0
15.8×10^{-5} M	1	89	10	0	0	0

The p-nitrophenol was added 15 minutes before the eggs were due to go into 4 cells. There is, as the data show, a time lag before cleavage is blocked which is particularly evident with the weakest blocking concentration. With higher concentrations the block is more immediate. At the same time the initial rise in respiratory rate is of much shorter duration in such solutions.

Using as the optimum concentration that which gives the maximum stimulation without a subsequent decrease with time, we may compare the various substituted phenols. In Table I these optimum concentrations, $[\Sigma P]$, at pH 8.0, are given for 15 substituted phenols. The

amount of respiratory stimulation at the optimum is also listed. We shall not at this time attempt to compare the various compounds on the basis of magnitude of respiratory stimulation. The comparison on the basis of optimum concentrations must, as was previously pointed out, take into account the pH effect which showed that for

TABLE I

Concentrations required for optimum stimulation of rate of oxygen consumption in fertilized sea urchin eggs. Temperature = 20.0° C.
$[\Sigma P]$ = total concentration in solution at pH 8.0.
$[HP]$ = calculated concentration of undissociated molecules.
$[P^-_i]$ = calculated concentration of the anion inside the cell.

	$[\Sigma P]$	Max. resp. stimulation	pK	$[HP]$	$[P^-_i]$
	$M \times 10^3$	*per cent*		$M \times 10^{10}$	$M \times 10^4$
o-nitrophenol	373	37(?)	7.25†	2,780,000	62.2
m-nitrophenol	27.3	93	8.34†	1,870,000	3.4
p-nitrophenol	10.3	128	7.22†	146,000	3.5
2, 4-dinitrophenol	6.78	172	4.00†‡	67.8	2.7
2, 6-dinitrophenol	11.6*		3.59†	45.3	4.6
2, 4, 6-trinitrophenol	1090*	0	0.80‡	6.9	440
o-chlorophenol	47.2*		8.50§	3,590,000	4.5
m-chlorophenol	69.9	71	8.85§	6,130,000	3.5
p-chlorophenol	181	30	9.18§	17,000,000	4.5
2, 4-dichlorophenol	23.5	131	7.75§	876,000	6.2
2, 5-dichlorophenol	12.3	73	7.35§	224,000	3.6
2, 6-dichlorophenol	991*	0	6.80§	5,880,000	371
3, 5-dichlorophenol	20.8	39	7.92§	943,000	4.5
2, 4, 6-trichlorophenol	6.9*		6.00†	6,870	2.7
2, 6-dichloro-4-nitrophenol	4.87	140	3.70‖	24.4	2.0

* Value determined from cleavage block data, i.e., concentration just below blocking.
† From the International Critical Tables.
‡ From Scudder (see bibliography).
§ From Murray and Gordon (see bibliography).
‖ From Bader (see bibliography).

a given substituted phenol, the same effect (e.g., optimum respiratory stimulation) was obtained at different pH's when there was the same calculated concentration of undissociated molecules present. The comparison of the different compounds is therefore first made on the basis of the concentration of undissociated molecules, $[HP]$, present in the solution at total concentrations giving optimum respiratory stimulation. These values are listed in the fourth column of the table, and it is evident that there are enormous differences between the different compounds. If the undissociated form were considered to be active (as the pH effect might indicate) then it would mean, for ex-

ample, that 700,000 molecules of p-chlorophenol are required to do the same job as one molecule of 2,6-dichloro-4-nitrophenol. Since this appeared extremely unlikely, a comparison of the various compounds was made on the basis of the calculated concentrations of anions, $[P^-_i]$, inside the cell at total concentrations giving optimum respiratory stimulation. The assumptions involved in this calculation are given in the introduction. These values are listed in the last column of Table I and it may be seen that with the exception of three compounds the values are all of the same order of magnitude. Two of the exceptions; namely, 2,4,6-trinitrophenol and 2,6-dichlorophenol give no respiratory stimulation, the $[P^-_i]$ values being obtained from cleavage block data. The other exception, o-nitrophenol, gave a small stimulation of respiratory rate. But our sample of this compound was probably impure as judged by its melting point. Dr. Clowes has suggested that the impurity may well be p-nitrophenol, which would account for the slight respiratory stimulation.

The three exceptions may therefore be omitted since they are not respiratory stimulants. The same may possibly hold for o-chlorophenol which was not sufficiently investigated to determine whether or not it will stimulate the respiratory rate. The narrow range in which the values of $[P^-_i]$ lie contrasts strikingly with the great divergence in $[HP]$ values of the various substituted phenols. Of the two alternatives, the anions $]P^-_i]$ or the undissociated molecules $[HP]$, the evidence shows that it is the former that must be considered the active form inside the cell.

The calculated $[P^-_i]$ values for different substituted phenols vary, the extremes showing a threefold variation. The data, however, are not sufficiently accurate for the differences to be considered significant. There are various sources of errors in the data. The errors in the manometric method are probably negligible. Those involved in the pH determinations and in slight variations in the course of a run are negligible for compounds with high pK values but not for compounds with low pK values. The pK values which are taken from the literature (see superscripts in Table I) are themselves subject to some error. This, as was shown in the introduction, affects principally the $[P^-_i]$ values for those compounds with high pK. In addition, certain of these compounds, particularly the mono- and dichlorophenols, are rather volatile, so that during the course of a run a certain amount of distillation into the alkali well of the vessel occurs. The magnitude of this factor is difficult to assess. It would, of course, tend to make the calculated $[P^-_i]$ values too high.

Effect of Changing the Internal pH

If the anion is the active form within the cell, then by increasing the internal pH (keeping the external pH constant) a lower total concentration of the substituted phenol should be required to give the optimum respiratory stimulation or the reversible block to cleavage. It is possible to increase the internal pH by the addition of a weak penetrating base such as ammonia. The pH change is readily demonstrated by first staining the eggs with neutral red, and, after washing, immersing them in the ammonia solution. The extent to which the red color fades (neutral red changes from red to yellow between pH 6.8 and pH 8.0) gives the increase in internal pH that has occurred.

The effect of increasing the internal pH on the concentration of dinitrophenol required for reversible cleavage block was investigated with eggs of *Urechis*. The solutions were prepared from carbonate-

Table II

Cleavage inhibition of Urechis eggs with 2, 4-dinitrophenol in ammoniacal sea water. Experimental and control solutions both prepared from carbonate-free sea water and buffered at pH 8.43 with glycylglycine.

Concentration of 2, 4-DNP	Cleavage in ammonia solution	Cleavage in control solution
(Molar)	(*per cent*)	(*per cent*)
0	99	99
2.6×10^{-4}	95	95
3.9×10^{-4}	0	95
6.0×10^{-4}	0	90

free sea water using the glycylglycine buffer and adjusting the pH with ammonia. In the control solutions NaOH was used instead of ammonia. It was found in the first place, that when sufficient ammonia was present to effect an increase in internal pH, cleavage was interfered with or proceeded abnormally. These same solutions when used on unfertilized eggs gave artificial parthenogenesis. With solutions weak enough in ammonia to have no effect on cleavage and no activating effect on unfertilized eggs there was no observable effect on the internal pH. We were unable to prepare an ammonia solution which would give an observable increase in internal pH without at the same time affecting cleavage or activating unfertilized eggs.

It was possible, however, to demonstrate, with the weakest ammonia solutions that give an observable increase in internal pH, an effect on the concentration of dinitrophenol required for cleavage block. The results of such an experiment are given in Table II. The

percentage of eggs that divided in the ammonia solution is seen to be as high as in the control. The cleavage here was rather abnormal (in regard to size of blastomeres) and only abnormal bottom-swimming embryos were obtained as compared with 90 per cent top swimmers in the controls. It may be seen from the table that the concentration of dinitrophenol required to block cleavage is considerably less in the ammonia solution than in the control. This result conforms then to the expectation, on the basis of the anion being the active agent inside the cell. The fact that the ammonia solution employed did not give normal later development does not seriously interfere with the interpretation, since we would not expect a summation effect with dinitrophenol.

Buffer Capacity of Eggs

One of the assumptions on which the calculation of the concentration of anions inside the cell is based is that the internal pH of the cell does not change in the solutions used. Since the substituted phenols are weak acids and penetrate only in the undissociated form,[3] an acidification of the interior might be expected to occur. This would be independent of the pH of the external solution and the extent of acidification should depend upon the total concentration of the substance. The amount of decrease in internal pH that would be expected would also depend upon the buffer capacity of the egg and the dissociation constant of the particular substituted phenol. We have attempted to settle this point by staining eggs with indicators that have their color change in the region of pH 4 to 6, but have been unable to find any that penetrate readily.

In place of this, a determination of the buffer capacity of egg material was made. For this determination eggs of *Strongylocentrotus* were first frozen at − 72° C. for several hours and rapidly thawed to room temperature. This completely breaks up the eggs, resulting in a fine granular suspension which we shall refer to as egg-brei. An egg brei containing originally 2 parts of eggs to one part of sea water by volume showed a pH of 6.35. In order to allow the suspension to be more easily stirred during titration, it was diluted with an equal volume of distilled water, which resulted in a slight rise in pH to 6.41. The titration curve of this diluted egg brei is shown in Fig. 2 (curve *A*), and a titration curve of ordinary sea water is given for comparison (curve *B*). In the 10 cc. of egg brei that was titrated there was $1\frac{2}{3}$ cc. of sea water along with the $3\frac{1}{3}$ cc. of egg material. Comparison

[3] Beck and Chambers (1937) have recently presented evidence to show that salts of weak fatty acids can under certain conditions penetrate marine eggs.

of curves *A* and *B* (the latter is for 5.0 cc. of sea water) shows that the correction for the sea water present is negligible. The buffer capacity of the egg brei is evidently quite high as the figure shows. To lower the pH from 6.4 to 5.4, 1.65 cc. of 0.1 normal acid are required. For the same buffering action a univalent acid with a pK of 6.4 would have to be present in an initial concentration of 0.04 molar, or considering the eggs alone, 0.12 molar.

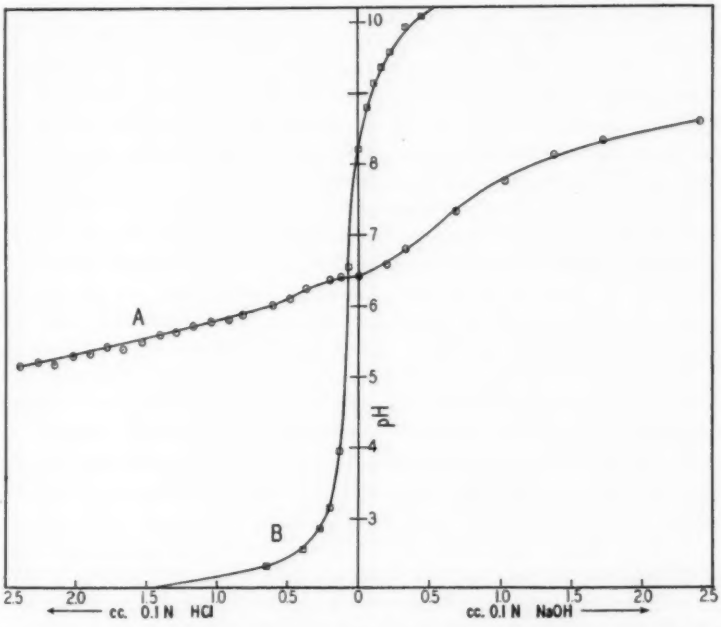

FIG. 2. Curve *A;* titration of 10 cc. of a suspension of sea-urchin egg brei (prepared by freezing at −72° C.) containing 3⅓ cc. of egg material, 1⅔ cc. of sea water, and 5 cc. of distilled water. Curve *B*; titration of 5 cc. of sea water.

We cannot, of course, assume that the buffer capacity determined in this manner gives a true picture of the situation in the living egg. Nevertheless, it would not be expected to be radically different. It is interesting to note that the egg brei shows a pH of 6.4 which is not very different from the value of 6.6 obtained by micro-injection of indicators (Needham and Needham, 1926).

For raising the internal pH with ammonia, at an external pH of 8, the total concentration of ammonia must be greater than 0.01 molar.

The optimum *total* concentrations of the different substituted phenols are all much lower than this.

It may be concluded, then, that the egg is sufficiently buffered to maintain a fairly constant pH in the presence of the concentrations of the substituted phenols employed in these experiments.

Discussion

In two recent articles, Krahl and Clowes (1938) present some tentative conclusions of their investigations on the action of substituted phenols. They are in agreement with our view (T. and H., 1937*b*) that penetration is effected in the undissociated form. Also, respiratory stimulation below the optimum is stated to depend upon the anion concentration inside the cell. But cleavage block and initial respiratory decrease (beyond the optimum), is considered to depend upon the concentration of undissociated molecules. The amount of stimulation at the optimum is then, according to these investigators, dependent upon the concentration of anions attained when the limiting concentration of molecules is reached, in the case of any given substituted phenol. The data obtained by Krahl and Clowes and by us agree, on the whole, very well. But in the experiments on the effect of changing the internal pH of the cell we differ. They find practically no effect on the total concentration required for cleavage block, whereas we find an effect in the direction expected according to our interpretation. Since the evidence from this source is partly responsible for the divergence in our respective views, a thorough investigation of the effect of changing the internal pH would be desirable. The difficulties involved in such experiments have been indicated above.

Field, Martin, and Field (1935, 1936) have compared the activity of several substituted phenols in stimulating yeast respiration. With those compounds for which dissociation constants were available, the calculated optimum concentrations of the undissociated form are given, since that is considered the active form by these authors. We have calculated from their data the concentration of anions that would be present inside the cell, using Gutstein's (1933) value of 6.2 for the pH of yeast. These values differ almost as much as do the figures for the optimum concentration of the undissociated form of the different substituted phenols that they list. It appears then that the situation in yeast is different from that in the marine eggs. It may be noted that the optimum concentrations of undissociated molecules obtained with yeast are much higher than with eggs, ranging from about 10 times as much in the case of the mono-nitrophenols to 1000 times as much with the dinitrophenols. It is not feasible at present to attempt

to resolve the differences between yeast and marine eggs. It is evident, however, that the wide differences in [*IIP*] obtained with the various substituted phenols in yeast do not support the view that the undissociated form is active.

Recently Bodine and Boell (1938) have reported experiments on grasshopper embryos in which no pH effect is manifest with suboptimal concentrations of dinitrocresol. Actually their curves show a slight increase in respiratory stimulation as the pH is lowered, but the effect is much too small to be interpreted simply on the basis of the variation in concentration of undissociated molecules. Possibly in the grasshopper egg the changes in external pH produce proportionate changes in internal pH. This would, in the case of substituted phenols with relatively large dissociation constants (see page 211) tend to result in a constant or only slightly varying $[P^{-}_{i}]$ and would, according to our view, explain the absence of a pH effect on the respiratory stimulation. The more marked effect which they obtained with a stronger than optimum concentration might be similarly explained, especially since the stimulation drops off quite rapidly with concentrations above the optimum.

The differences in $[P^{-}_{i}]$ shown in Table I do not seem to us to be significant, for the reasons stated above. It appears likely, then, that the different substituted phenols have the same activity, considered on the basis of the $[P^{-}_{i}]$ required to produce the same effect. This is consistent with the view previously expressed by Clowes and Krahl (1936) that these compounds owe their activity to the phenolic OH group. The magnitude of the respiratory stimulation would depend then on the effect on the phenolate ion of the type of substitution in the benzene ring.

Summary

1. Data on the concentrations required for optimum respiratory stimulation and reversible cleavage block in fertilized sea urchin eggs are presented for fifteen different nitro- and halophenols.

2. The experiments show that it is necessary to define the optimum as the highest respiratory increase that does not diminish with time.

3. Cleavage block occurs with concentrations just beyond the optimum defined in this manner.

4. Comparison of the calculated concentrations of undissociated molecules and of anions inside the cell for the different substituted phenols supports the previously expressed view that the anion is the active agent. Penetration is accomplished in the undissociated form, as previously shown.

5. The results of experiments in which the internal pH is raised also support this view.

6. Titration curves of egg brei indicate a high buffer capacity for the egg and support the assumption that the internal pH does not change in the presence of the substituted phenols.

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THE COPPER CONTENT AND COPPER TOLERANCE OF SOME SPECIES OF MOLLUSKS OF THE SOUTHERN CALIFORNIA COAST

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This report is mainly concerned with the results of analyses of specimens of some species of marine mollusks with reference to the variation of copper content with age or size, and the approximate upper limits of copper concentration in sea water that a few of them can tolerate.

Some quantitative determinations of the copper content of marine animals have previously been reported, particularly by Hiltner and Wichmann (1919), by Rose and Bodansky (1920), by Severy (1923), and by Galtsoff and Whipple (1931). These investigators found wide variations in the copper concentration of some of the species they studied, and Hiltner and Wichmann (1919) point out that both copper and zinc can be absorbed and retained in the tissues of oysters in much larger quantities than is needed for normal metabolism. Prytherch (1931) obtained evidence that copper is required for the attachment, metamorphosis, and survival of the oyster. Orton (1924) examined the English oyster for this element and mentions the poisonous effects on marine life of excess copper in sea water.

Copper occurs in combination as the chromoprotein hemocyanin in many species of arthropods and mollusks. For a review and bibliography of the work concerning this respiratory pigment, the reader is referred to the paper by Redfield (1934). Elvehjem (1935) has reviewed and discussed the biological significance of copper.

METHODS

The applicability of the sodium diethyldithiocarbamate method of Callan and Henderson (1929) to the determination of copper in biological materials has been established by McFarlane (1932) and by Tompsett (1934*a*, *b*, *c*, 1935). In this investigation, the procedure adopted was similar to theirs.

Water, redistilled in an all-Pyrex glass apparatus, was used for preparing reagents and rinsing containers. The reagents employed were tested and found copper-free. Tissues were ashed in silica vessels

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that had previously been treated with sodium acetate. The moist tissue was dried at 105–120°, weighed, and ashed in a muffle furnace at 380–420°. When necessary, small quantities of dilute nitric acid were added to hasten the oxidation of the carbon. A few grams of sodium phosphate were usually mixed with the dry tissue before ashing. The ash was treated with 6 N hydrochloric acid, or first with the 6 N acid, and then with redistilled water. An aliquot portion of solution was transferred to a glass-stoppered graduated cylinder which contained small quantities of sodium pyrophosphate and 25 per cent sodium citrate solutions. Both 4 per cent and saturated solutions of sodium pyrophosphate were used in the course of this work, and the volumes employed depended on the estimated quantities of iron present in the unknowns. The solutions in the cylinders were finally made alkaline to litmus with ammonium hydroxide. The standards contained 0.10 or 0.20 mg. of copper as cupric sulphate besides the other reagents.

After cooling, 1 ml. or 2 ml. of a 2 per cent sodium diethyldithiocarbamate solution was added and the mixture shaken, and then 10 ml. or integral multiples thereof of benzene, toluene, xylene, or ligroin was introduced by pipette for extraction of the colored complex. The whole was then vigorously agitated. It was somewhat difficult to recover the solvent layer completely, or in the same quantities from standard and unknown. Therefore, the same fractional amounts were removed by pipette from each, and the colorimetric determination made. This procedure is correct if the quantities of organic solvent dissolved in the solution containing the standard and that to be tested are the same. Since n-amyl alcohol, which was used by McFarlane (1932), is fairly soluble in water, the other solvents which are much less soluble were substituted for the alcohol. Thus a possible error was avoided. Any water or precipitated material in the solvent layers was removed by centrifuging for a minute or two.

Since a few grains of sand were sometimes found present in the ash, even though considerable effort was made to remove them all from the moist tissues, these may have been a source of error. However, when small samples of sand, taken from the beach where many of the mollusks were obtained, were mixed with sugar charcoal and the above procedure carried out, the quantity of copper found was negligible. Although this is not evidence that such would always be the case, errors thus produced would be expected to be small.

According to Griffiths (1892), manganese is found in the respiratory pigment of the mollusk, *Pinna squamosa*. It is quite probable that this element occurs in other species of mollusks, and it was thus de-

sirable to determine whether or not it could cause discrepancies in the analyses.

It was found that the reddish product formed when manganous chloride or sulphate react with sodium diethyldithiocarbamate is not readily extracted from the aqueous layer by benzene or toluene unless an electrolyte, such as, for example, HCl, NaCl, NH_4OH, is present. However, sodium pyrophosphate was found to form a non-ionizable compound with manganese as it does with iron when the solution is made basic with ammonium hydroxide. An alkaline solution may contain as much as 2.0 mg. of manganese and only .10 mg. of copper, but if sufficient pyrophosphate is present, no appreciable error results in the colorimetric determination for copper.

Zinc, which is also known to be present in mollusks, does not form a colored complex with the carbamate reagent. According to Callan and Henderson (1929), this element may be present in as high a concentration as 0.1 gram per 100 ml. and will not interfere with the copper determination provided sufficient ammonia is present and not too much of the colorimetric reagent has been introduced.

TABLE I

Copper recovery experiments

Series	Wt. of moist tissue	Quantity of copper added	Total copper found	Copper in tissue
	grams	*mg.*	*mg.*	*mg.*
A	30	0	0.158	0.158
	30	0.020	0.173	0.153
	30	0.060	0.211	0.151
B	30	0	0.071	0.071
	30	0.050	0.102	0.052
	30	0.080	0.139	0.059

COPPER RECOVERY EXPERIMENTS

Representative data obtained in a series of copper recovery experiments are given in Table I. In each series, the tissues of a number of mussels, *Mytilus californianus*, of various sizes were minced, drained fairly free of fluids, and well mixed. Portions were then weighed out. Copper was introduced by means of a standard cupric sulphate solution containing 0.10 mg. of copper per ml. In the second series of experiments, 3 grams of anhydrous sodium phosphate was added to each vessel. No sodium phosphate was used in the others, but good recoveries resulted.

TABLE II

The copper content of mollusks

Species	Length or weight of animals	Mg. of Cu per 100 grams of moist tissue	Mg. of Cu per 100 grams of dry tissue
Archidoris montereyensis	30.5 grams (one animal)* [a]	0.13	0.59
Astraea undosa (wavy top)		0.80	2.83
		1.26	4.34
Chione undatella (cockle)	2.7–3.9 cm., mean 3.6 cm. (7 animals) [b]	0.12	0.94
		0.20	1.24
Donax gouldii (bean clam)		0.06	0.41
		0.07	0.44
Helix aspersa (common terrestrial snail)	0.1–1.5 grams	1.63	10.7
	4.7–7.6 grams	10.8	56.1
Ischnochiton conspicuus (conspicuous chiton)	22–32.5 grams	0.37	1.03
		0.25	0.97
		0.28	1.08
Navanax inermis (striped mollusk)	51 grams (one animal) [c]	0.44	4.98
Mytilus edulis (bay mussel)		0.23	1.17
		0.37	2.38
Paphia staminea var. *laciniata* (rock cockle)		0.24	1.31
		0.18	1.48
	4.0 ± 0.2 cm.	0.21	1.68
	5.2 ± 0.1 cm. (three animals) [d]	0.18	1.44
Pecten circularis aequisulcatus (speckled scallop)	5.5–6.5 cm. [e]	0.31	2.40
Tegula gallina (speckled turban)		0.61	2.03
		0.61	2.30
Tegula viridula var. *ligulata* (banded turban)		0.81	3.25
		0.92	5.93
Tivela crassatelloides (clam)	[f]	0.21	1.19
		0.23	1.39

* Amount of copper per animal: [a] 0.039 mg.; [b] 0.006 mg.; [c] 0.213 mg.; [d] 0.009 mg.; [e] 0.043 mg.; [f] 0.013 mg.

Results

For a description of the species investigated, refer to Johnson and Snook (1927). Some general data are given in Table II. In this instance, the tissues of from one to twenty animals were usually employed in an analysis without reference to the size of the organisms. Where ranges of dimensions are given, they represent the maximum for the shells. The best basis for comparison of results is that of the dry

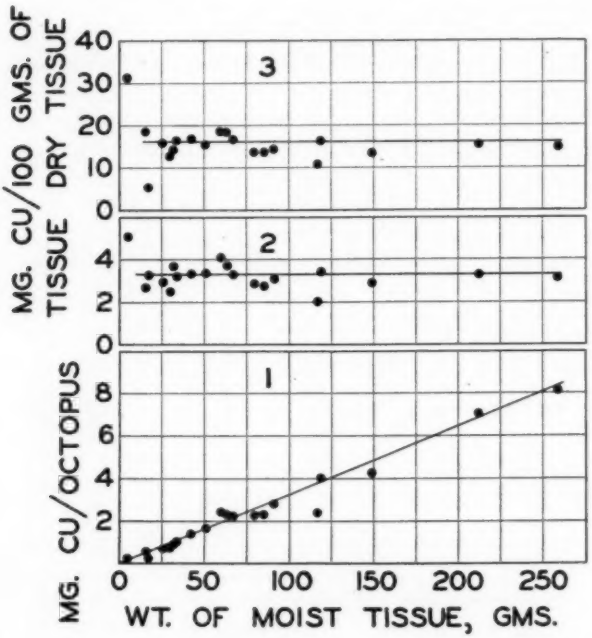

FIG. 1. Variation of copper content with size in the octopus, *Polypus bimaculatus*. Chart 1, mg. of copper per octopus; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

tissue, since it was generally not possible to remove the excess sea water completely from the moist tissues without endangering the loss of body fluids. Even heat-dried tissue may have caused inconsistencies if the animals had different proportional amounts of oxidizable lipoids, fats, and allied substances. This factor was believed to be less serious than those which would have to be reckoned with in the use of wet weights. A vacuum oven was not available. Concentration, in this

case, means the calculated copper content per unit weight of tissue and does not imply a homogeneous distribution.

Severy (1923) found no copper in the Pacific coast clams, *Ensis americanus* and *Venus kennicottii*. However, the writer found copper in small concentrations in both *Donax gouldii* and *Tivela crassatelloides*.

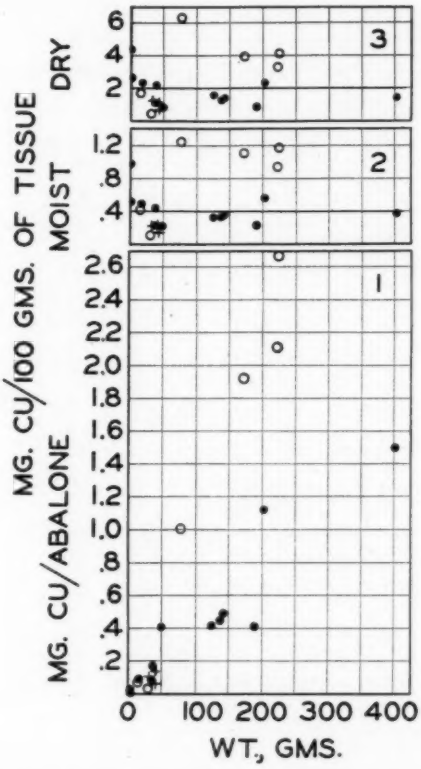

FIG. 2. Variation of copper content with size in the abalones, *Haliotis fulgens*, ○ = *H. cracherodii*, + = *H. rufescens*. The abscissa represents the weight of the moist tissue of each individual. Chart 1, mg. of copper per abalone; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

It was of interest to determine the copper content of animals of different sizes of a few species and to learn whether or not there is any change in the copper content per unit weight of tissue with age or size. Data for six species are plotted in Figs. 1 to 6. Each plotted point

represents the results of an analysis on a single individual with the exception of the data concerning the California sea mussel. External shells were excluded in the analyses.

The copper content in mg., s, of the octopus, *Polypus bimaculatus*,

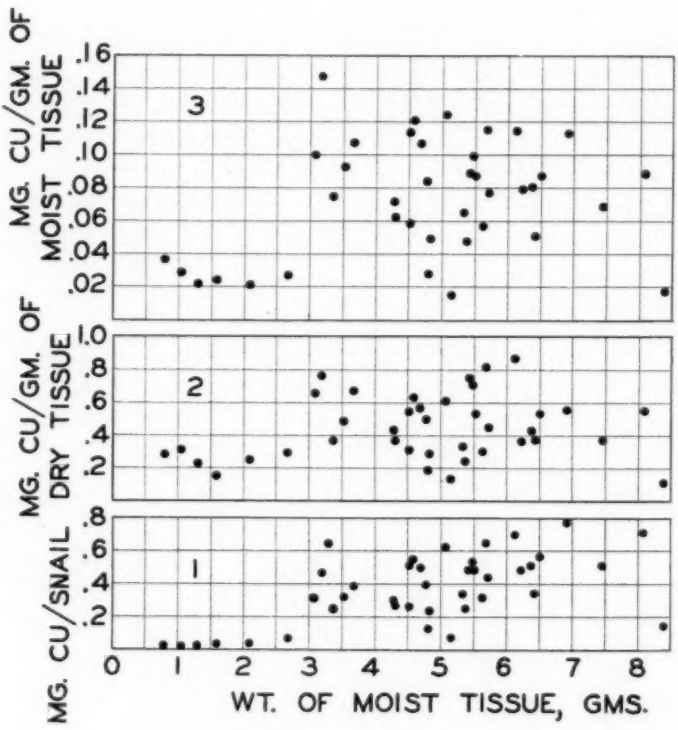

FIG. 3. Variation of copper content with size in the terrestrial snail, *Helix aspersa*. The abscissa represents the weight of the moist tissue of each individual. Chart 1, mg. of copper per snail; Chart 2, mg. of copper per gram of dry tissue; Chart 3, mg. of copper per gram of moist tissue.

varies linearly with the weight in grams, m, of the animal (Fig. 1) and is given by the equation, $s = 0.031\ m$. Mean values of the copper concentration are 3.15 mg. of copper per 100 grams of moist tissue, and 15.6 mg. of copper per 100 grams of dry tissue. The data indicate that these concentrations are constant over the range investigated.

Data concerning the species of abalones, *Haliotis fulgens*, *H. cracherodii*, and *H.* rufescens are plotted in Fig. 2.

Analyses were made of specimens of the terrestrial snail, *Helix aspersa*, for comparative purposes, and results are plotted in Fig. 3. These animals were collected in the late winter and early spring during a rainy period and, for the most part, were not in the resting stage at the time of collection. They were usually kept in the laboratory a few days prior to the analysis. The copper content of individuals which had approximately the same weight was found to be quite variable.

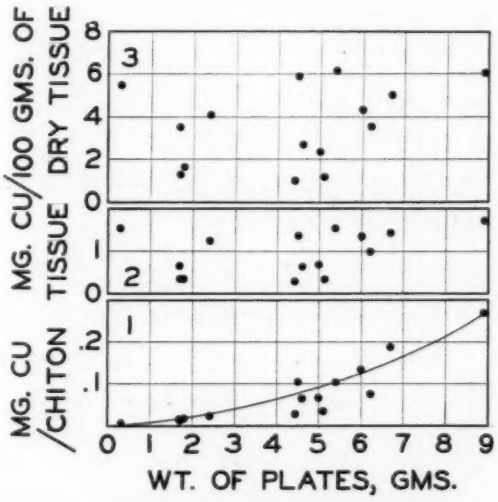

FIG. 4. Variation of copper content with size in the chiton, *Ischnochiton conspicuus*. The abscissa represents the weight of the plates of each individual. Chart 1, mg. of copper per chiton; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

The copper concentration in both moist and dry tissues increases with increase in size of the animals. This fact is also shown by the data in Table II which were obtained by analyzing two groups of animals of a smaller and a larger definite weight-range. Severy (1923), likewise, found a much higher copper concentration in older specimens of the terrestrial mollusk, *Limax maximus*, than in less mature ones. Both the octopus and the snail contain sufficient copper to cause the ash of these animals to be quite blue.

Results of analyses for copper in the chiton, *Ischnochiton conspicuus*, and in the sea hare, *Tethys californica*, are given in Figs. 4 and 5, re-

spectively. It is not known whether or not hemocyanin is the respiratory pigment in these species, whereas it is hemocyanin in the octopus and the snail, but in view of the relatively small amounts of copper in the sea hare it seems rather unlikely in this instance. Because of the difficulty of completely removing excess sea water from the chitons, the weight of the plates of each animal has been used as a measure of size.

The copper concentration in the dry tissue of the California sea

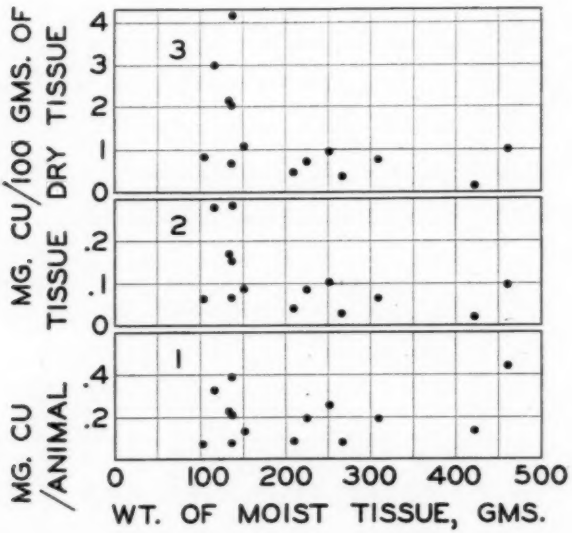

FIG. 5. Variation of copper content with size in the sea hare, *Tethys californica*. The abscissa represents the weight of each animal. Chart 1, mg. of copper per sea hare; Chart 2, mg. of copper per 100 grams of moist tissue; Chart 3, mg. of copper per 100 grams of dry tissue.

mussel falls off with increase in length (Fig. 6) of the valves. In general a number of animals were used in each analysis, and these were selected so that they had the same length within ± 0.5 cm. of the mean for the larger sizes and ± 0.2 cm. for the smaller sizes. Selection was made by using length of valves rather than the weight of the moist tissue because the procedure was then more rapid and more accurate. The weight of the moist tissue cannot be determined with the same accuracy as the length of the valves because of the accompanying sea water.

An investigation was made of the variation of copper content and concentration in different groups of organs obtained from young mussels and more mature ones, and data are given in Table III. The tissues of these animals were first hardened in a 2 per cent formaldehyde solution before removal from the valves. Since both the gonads and mantle contain eggs or sperm, these organs were grouped together. The foot is largely muscular tissue. No attempt was made to separate males from females. The decrease in copper concentration with increase in size is to be noted.

Chou and Adolf (1935) examined certain organs of five cadavers for

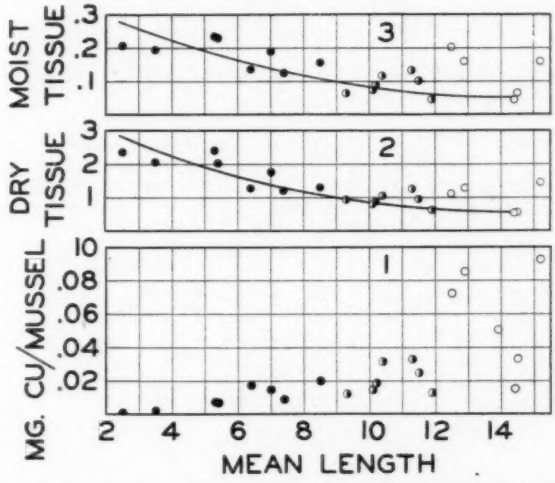

FIG. 6. Variation of copper content with size in the California sea mussel, *Mytilus californianus*. The abscissa represents the mean length of the valves when a number of animals were used. ○, one animal; ◑, 2–3 animals; ●, 5 or more animals. Chart 1, mg. of copper per mussel (mean values); Chart 2, mg. of copper per 100 grams of dry tissue; Chart 3, mg. of copper per 100 grams of moist tissue.

copper and found a markedly higher concentration in the infants than in the adults. Sheldon and Ramage (1931) analyzed five foetal livers of man for copper and found a concentration 5 to 7 times that in adult organs. Perhaps copper plays a significant rôle in growth metabolism in many animals.

COPPER CONTENT OF SEA WATER

In connection with the work concerning the copper tolerance of certain mollusks, it was desirable to know the copper content of the ocean water in which they live.

Using a procedure similar to that of Atkins (1933), determinations were made of the copper concentration in surface water taken 1,000 feet from shore at this station. The water was collected in a glass bucket and stored in Pyrex containers. Cursory examinations, made by adding 1 ml. of 2 per cent sodium diethyldithiocarbamate solution to 1 liter of sea water, extracting with 10 ml. of carbon tetrachloride

TABLE III

The copper content of groups of organs of the sea mussel, *Mytilus californianus* *

Length of mussels	No. of mussels used	Hepatopancreas, intestines and heart	Mantle and gonads	Muscles and foot	Gills and palps
cm.					
Range: 6.3–7.5	30	(*a*) 0.0038	0.0032	0.0022	0.0047
Mean: 6.9		(*b*) 27.4	23.0	15.8	33.8
		(*c*) 1.34	0.20	0.36	0.65
		(*d*) 9.35	2.52	2.26	7.70
Range: 6.6–7.7	30	(*a*) 0.0049	0.0034	0.0025	0.0052
Mean: 7.1		(*b*) 30.6	21.2	15.6	32.6
		(*c*) 1.66	0.20	0.36	0.55
		(*d*) 13.6	2.79	2.44	7.07
Range: 11.9–13.0	5	(*a*) 0.0159	0.0216	0.0042	0.0148
Mean: 12.5		(*b*) 28.2	38.2	7.4	26.2
		(*c*) 0.86	0.21	0.14	0.39
		(*d*) 8.70	3.26	1.00	5.44
Range: 11.8–12.4	4	(*a*) 0.0172	0.0234	0.0039	0.0102
Mean: 12.1		(*b*) 31.5	42.8	7.1	18.6
		(*c*) 0.85	0.23	0.12	0.27
		(*d*) 8.21	1.65	0.57	3.66

* The data for each group of organs of each series is placed in the following order:
(*a*) mg. of copper per group of organs per mussel (mean values),
(*b*) per cent of total copper present per mussel,
(*c*) mg. of copper per 100 grams of moist tissue,
(*d*) mg. of copper per 100 grams of dry tissue.

or benzene and estimating colorimetrically, showed that the copper present could not be greater than 0.004 mg. per kg.

Twenty liters were electrolyzed, 1 liter at a time, between platinum electrodes at a potential difference of approximately 2 volts for at least 8 hours, and at a temperature of 70–90° during much of this period. The copper was dissolved from the cathode with dilute copper-free nitric acid, the solution was then neutralized with ammonium hydroxide, cooled, 1 ml. of the carbamate reagent introduced and the total extracted with benzene. A colorimetric determination showed

0.0010 mg. of copper present per kg. of sea water (salinity 33.68 per cent). An analysis of a 10-liter sample taken six weeks later showed 0.0014 mg. of copper present per kg. (salinity 33.63 per cent). There had been no rain in this region for several months.

Copper Tolerance of a Few Species of Marine Mollusks

The approximate upper limits of copper concentration that some mollusks can tolerate, when kept in the laboratory under conditions to be described, were determined as follows.

Table IV

The copper tolerance of marine mollusks

Mg. of copper added per kg. of sea water		Time of survival, days				
		0	0.05	0.10	0.15	0.20
Species	Animals per jar					
Acmaea scabra var. *limatula*	4	16†		3†		3†
Fusinus kobelti	2–3	60		60		8†
Astraea undosa	2	30	14†	5†		5†
Haliotis fulgens	1–2	30	30	3†		
Ischnochiton conspicuus	2–3	60		60	10†	3†
Mytilus californianus	2	60		60	30	2†
Mytilus edulis	3	35		35		17†
Tegula gallina	3–4	60	60	15†		8†
Tegula viridula var. *ligulata*	4	60	60	25†		18†
Paphia staminea var. *laciniata*	3–4	100 alive at end of 30 days when 1.0 mg. of Cu added per kg. One animal alive at end of 65 days when 3.0 mg. of Cu added per kg.; another lived 58 days in latter concentration.				

† Dead at end of this time. All others alive at end of stated period.

In a series of jars, each of which contained 8 kg. of sea water, were placed a number of mollusks of a given species. Definite amounts of a standard cupric chloride solution containing 0.08 mg. of copper per ml. were then added to each jar. A continuous stream of air was passed in to keep the water well aerated, and distilled water added every day or two so that the volume and salinity were maintained constant. Many of the experiments were run in duplicate. Controls, to which no copper had been added, were run simultaneously. Temperature variations corresponded approximately to those of the sea. In general,

there was a growth of algae, bacteria, etc. so that the animals were not without food. However, it should be pointed out that the natural habitat was not completely duplicated and other factors may have contributed to a decrease of the life span, such as the effect of added copper on the growth of certain organisms used for food.

Results are presented in Table IV. The upper limit of copper tolerance for most of the species investigated lies in a range of 0.10 to 0.20 mg. of added copper per kilogram of sea water and thus is about 100 to 200 times that normally present. However, the clam, *Paphia staminea* var. *laciniata*, can tolerate a much higher concentration. The addition of 3.0 mg. of copper as cupric chloride to a kilogram of sea water produces a noticeable cloudiness. Further introduction of copper alters the concentration of some of the other constituents because of precipitation.

Since younger mussels have a higher copper content per unit weight of tissue than more mature ones, it was thought that the former might tolerate 0.20 mg. of added copper per kilogram, but this was found to be untrue. The valves of the animals in this instance had a mean length of 5.3 cm.

Summary

The copper content and concentration of several species of mollusks has been determined with particular reference to variation with age or size. In the snail, *Helix aspersa*, the amount of copper per unit weight of tissue increases with increase in size, whereas it decreases in the California sea mussel. In the octopus, *Polypus bimaculatus*, it remains constant. Samples of sea water of the Pacific were found to contain 0.001 mg. of copper per kilogram. The upper limits of copper tolerance of a few species of marine mollusks, when kept in the laboratory under given conditions, lies in the range from 0.10 to 0.20 mg. of added copper per kilogram of sea water. The clam, *Paphia staminea* var. *laciniata*, can tolerate a much higher concentration than the above.

I am indebted to many of the members of the staff of the Scripps Institution and to others for their helpful suggestions and kindly interest in this work and for their aid in the collection and identification of specimens.

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THE EFFECTS OF ULTRA-VIOLET RADIATIONS OF VARIOUS WAVE-LENGTHS UPON CLEAVAGE OF SEA URCHIN EGGS

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In a preceding paper retarding effects of radiations of λ2537A upon cleavage of eggs of the sea urchin, *Strongylocentrotus purpuratus*, were reported (Giese, 1938). The following account, extending the above study to wave-lengths of 2654, 2804, 3025, 3130 and 3660A, attempts to determine (1) the amount of retardation, if any, produced by known dosages of the above wave-lengths; (2) the amount of acceleration, if any, following low dosages; and (3) the region of the spectrum most effective in producing the effects noted.

The source of radiations (a water-cooled quartz mercury arc), the monochromator with optical parts of natural quartz, and the thermo-pile-galvanometer system for measuring intensities—were the same as those employed in the previous study. The data on intensities and the dosage ranges employed are summarized in Table I.

The animals were collected during the winter breeding seasons of 1937 and 1938 and the eggs were obtained and handled in the manner previously described. Approximately 300 eggs were used for each test. The experiments here reported were performed on fertilized eggs, irradiation beginning approximately 30 minutes after insemination. A dosage series of approximately 1024, 256, 64, 16 and 1 second was given at each of the wave-lengths. Three series of experiments were performed at each wave-length.

In most cases eggs from a single female were irradiated with a series of graded dosages of a given wave-length. In a few cases eggs from a single female were used for a series of dosages at each of two different wave-lengths. Thus in one instance eggs from one female were used for experiments with radiations of both λ2654 and λ2804A. The differences in effects of irradiation in this and other comparable cases were similar to those obtained with eggs of different females, the differences being of second order.

Following irradiation the eggs were pipetted into 1″ Syracuse watch-glasses, approximately 100 per dish, and were kept at 15 ± 1° C. Examinations were made at half-hour intervals, an attempt being

made to obtain the time when a large proportion of the eggs had just completed a given cleavage. After the 64-celled stage had been attained observations were made every 12 hours. As in the previous study, so here, abnormal development of eggs was found to be only a small part of 1 per cent of the total number used. Controls always developed into plutei normal to all appearances, therefore the conditions were considered satisfactory for the investigation.

TABLE I

Intensity and dosage measurements

λ in A	Exper. Number	I in ergs/sec./mm.²	Maximum dosage failing to retard cleavage in ergs/mm.²	Total dosage range in ergs/mm.²
2537	1	1.24	37.2	1.07–1585
	2	1.15	34.5	
	3	1.07	32.1	
2654	1	3.19	—	2.76–3444
	2	2.84	45.45	
	3	2.76	44.2	
2804	1	2.86	—	2.80–3090
	2	2.86	—	
	3	2.80	—	
3025	1	4.06	32.5	4.06–6840
	2	5.10	81.6	
	3	5.10	—	
3130	1	20.7	166	11.48–21,200
	2	11.48	—	
	3	13.74	210	
3660	1	38.5	(39,400)*	27.7 –74,500
	2	27.7	28,300	
	3	44.3	(74,500)*	

* At these dosages retardation was observed, but too slight to be accurately determined.

EXPERIMENTAL

In none of the experiments for any of the wave-lengths and dosages tried was there indication of definite stimulation and acceleration of the division rate, a finding in agreement with previous studies at λ2537A. The minimum dosages used are given in Table I. The eggs irradiated with a dosage below the retardation threshold cleave comparably to controls within the limits of accuracy of the investigation.

It is, of course, possible that the exact dose required for stimulation was not given or that even the lowest dosage was too great to induce acceleration.

While at the smaller dosages there was no retardation of the cleavage rate, such retardation was evident with sufficiently large dosages, the action of different wave-lengths being different only in degree. In each case retardation did not occur below a certain threshold (Fig. 2). In Table I are given data on the maximum dosage tried which failed to retard cleavage.

Retardation of cleavage was found to increase with dosage at all of the wave-lengths, as might be expected. After intermediate dosages of radiation, cleavage was normal and rhythmic, but after large dosages not only was cleavage delayed but it was often irregular; for example, whereas controls showed at most two cleavage stages at any given time, those irradiated with large dosages might show three or even four stages at one time. Occasionally abnormal cleavages appeared as well. Therefore it was often difficult to collect data for the larger dosages. None of the dosages used, however, were adequate to completely prevent cleavage.

Retardation following prolonged irradiation was apparent not only during cleavage but also at later stages in development. Thus at the time the blastulae of the controls were rotating within the fertilization membranes, those sufficiently irradiated were still immotile; when controls hatched from the membranes, those irradiated were still confined; when gastrulae formed, those irradiated were only beginning invagination.

In all cases eggs irradiated for 1, 16, and 64 seconds gave rise to blastulae, gastrulae and plutei normal to all appearances; in most cases those irradiated for 256 seconds did so as well. However, only in experiments at wave-lengths of 2537, 3130 and 3660A did plutei develop when eggs were given the maximum dosages recorded in Table I. In the experiments with large dosages at other wave-lengths the blastulae were as a rule abnormal and gastrulated abnormally. When plutei-like larvae developed they were abnormal, although in some instances skeletal rods, differentiated gut and pigment cells appeared. Many of these eggs, however, developed into balls of motile cells which failed to develop further. When in other cases blastulae and gastrulae were formed, however, they were choked with cells and appeared much darker than controls.

For each experiment the number of divisions of the controls and irradiated eggs was plotted against the time since insemination as in

Fig. 1, an example of a dosage series at λ2804A. The dosage in ergs per square millimeter for each curve was determined from the intensity readings and added to the graphs. The difference in time of development to the 8-celled stage between the control and the irradiated eggs was determined from the graph. The retardation of this third cleavage was then plotted against the log of the dosage (in ergs/mm.²) for each of the wave-lengths to obtain a comparison of the efficiency of the various wave-lengths in retarding cleavage. The data are given in

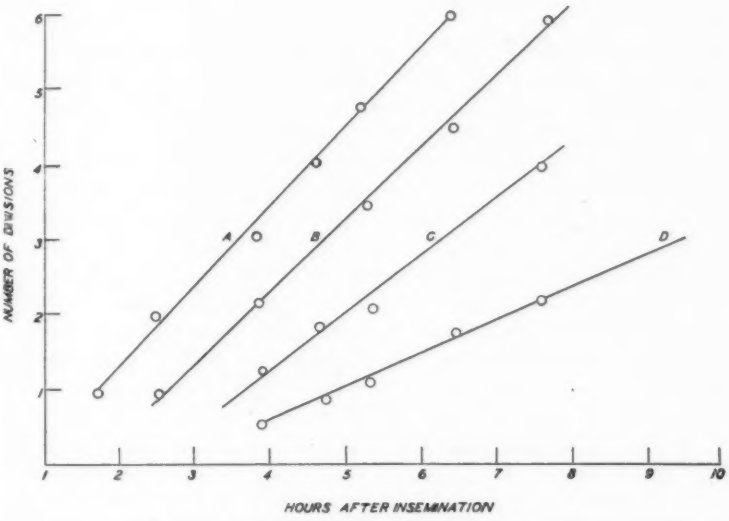

FIG. 1. Retardation of cleavage by irradiation with λ2804A, intensity 2.86 ergs/mm.²/sec.

A. Control.

B. Dosage 183 ergs/mm.²; retardation at 8-cell stage 1 hour and 8 minutes.

C. Dosage 1244 ergs/mm.²; retardation at 8-cell stage 2 hours and 42 minutes.

D. Dosage 3090 ergs/mm.²; retardation 5 hours and 48 minutes.

Fig. 2. The third cleavage was chosen for the comparison because (1) it is the midpoint of the developmental curves obtained; (2) it can be determined with greater accuracy than later cleavages; (3) it probably occurs after the latent period lag and by the time the effects of the irradiation have had full opportunity for expression. While the data are fairly homogeneous there is variability as detected in the spread of points on each side of the curves. It is possible that eggs from different females react to variable degrees. Part of the variation is undoubtedly

due to temperature changes and temperature differences for different experiments ($T = 15 \pm 1°$ C.). It is possible that minor errors in estimates and in other experimental procedures add further to the spread.

Examination of Fig. 2 discloses that the order of efficiency of the wave-lengths used, on the basis of incident energy, from the most effective to least is (1) 2804A, (2) 2537, 2654, 3025, (3) 3130 and (4) 3660. The difference between (1) and (2) is not large; the difference

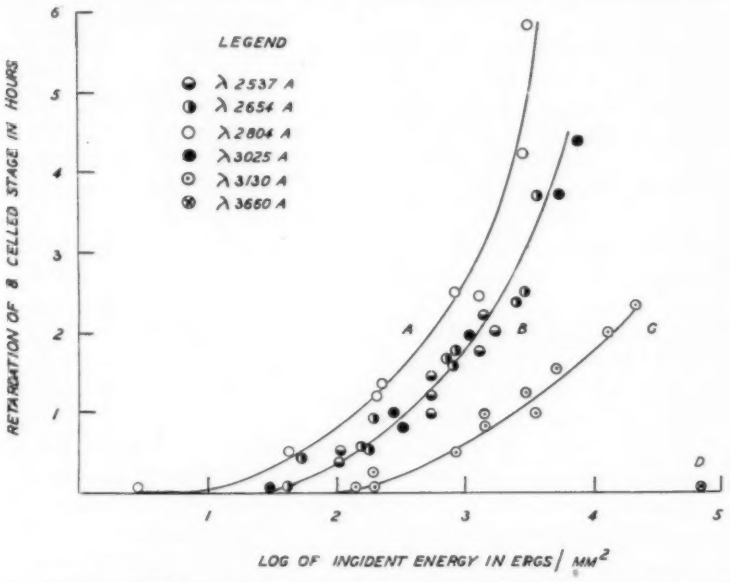

FIG. 2. Difference in efficiency of various wave-lengths in retardation of cleavage.

A. Curve drawn through points for λ2804A.
B. Curve drawn through points for λ's 2537, 2654, and 3025A.
C. Curve drawn through points for λ3130A.
D. λ3660A.

between (2) and (3), however, is great and between (3) and (4) very great. The difference in efficiency at the different wave-lengths may be due to the differences in absorption at the various wave-lengths. According to the Grotthus Draper law, only absorbed light is effective in promoting a photochemical reaction. If there is to be exact proportionality between absorption and effect, we should expect that the absorption at λ2804A would be about double that at λ2537, 2654 and 3025A and about 20 times that at 3130A.

Vlès and Gex (1928, 1934), using a quartz microscope and a spectroscope, measured the extinction of light by single eggs of the sea urchin, *Paracentrotus lividus*. They found for the fresh unfertilized egg a strong absorption band below λ2900A with a maximum at about λ2800A. At λ2537 and λ2654A the absorption was almost as great (the difference being of the order of one per cent), at λ3025A it had fallen off some 20 per cent, and only a little more to the beginning of the visible where another drop occurred. The fertilized egg was observed to have an absorption spectrum similar in general to that of the unfertilized egg (1928). Considerable variation was noted from egg to egg.

Unfortunately comparable equipment was not available to check the extinction by the eggs here used. However, the method previously used for paramecia was employed (Giese and Leighton, 1935*b*), readings of the galvanometer being taken first with the cell empty, then filled with unfertilized eggs, and the eggs counted. Three and in some cases four or five measurements were made at each wave-length, after several preliminary trials in which the eggs were not counted, and the averages are given in Table II. Considerable variation was observed for different samples of eggs. While the experimental error is large, as indicated by the standard deviations, the order of magnitude of the extinction is indicated, and such large differences as between λ2537 and λ2804A are significant. In all cases the differences of the averages of the groups from one another are many times the probable error, which is generally less than ± 0.5. In contrast to the data of Vlès and Gex, these data indicate a maximal extinction at λ2537A. Vlès and Gex found in most cases a decrease in absorption by normal eggs at this wave-length although in some of their cases this was not true. For eggs undergoing cytolysis due to dilution of sea water with distilled water they found a rise at λ2537A. That the method here used is not without merit is indicated by measurements on extinction of the ultra-violet light by sea urchin sperm. It was found that the maximum extinction occurred at λ2654A, a considerable decrease occurring at λ2804A, which is well in agreement with what might be expected of a cell made up mainly of nucleoproteins. However, it must be remembered that only part of the extinction is due to true absorption, the rest being due to scattering. With the experimental method here used the scattering would probably be a greater source of error than with the method employed by Vlès and Gex. Scattering varies inversely with some power of the wave-length as well as with the change in refractive index with wave-length (Teorell, 1930). If the change in the refractive index were negligible and the approximate value of the change in

scattering with wave-length found for *Paramecium* were to be used here (Giese and Leighton, 1935*b*), one could not explain the high extinction value at λ2537A as due entirely to a higher scattering. On the other hand, it is possible that the refractive index changes suddenly and that a corresponding increase in scattering occurs. The high value at λ2537A may, however, indicate a high absorption. Gates (1930) observed a higher absorption value at λ2537A than at λ2804A for both *S. aureus* and *B. coli*.

It is probable that in both sets of data the scattering masks the true absorption, but it is unlikely that the scattering is so great as to completely disguise differences of the order of magnitude needed to explain completely the differences in action of the different wave-lengths. It therefore seems more probable that it is not total absorption but rather selective absorption by some sensitive material which determines

TABLE II

Extinction of ultra-violet light by sea urchin eggs

λ in A	$1 - I/I_0 \times \frac{1}{A_e} \times 100$	λ in A	$1 - I/I_0 \times \frac{1}{A_e} \times 100$
2537	105 ± 5.50	3130	64.73 ± 8.00
2654	71.3 ± 4.40	3660	64.53 ± 9.32
2804	76.3 ± 7.78	5844	41.17 ± 4.21
3025	76.06 ± 5.54	5904	25.02 ± 2.44

I_0 = intensity of incident light, I of transmitted light.
A_e = fraction of the cell bottom occupied by eggs.
The whole expression gives the percentage of light incident upon the egg which is extinguished.

destructiveness of the rays. On this basis the substance absorbing λ2804A is most likely the sensitive substance, for approximately equivalent absorption is found at λ2654 and λ3025A, but their efficiency is less than that of λ2804A. The low efficiency of λ2537A may be due to the fact that radiations of this wave-length are so strongly absorbed at the exterior of the cell that they do not reach the seat of the sensitive material (Sonne, 1929).

Considering the high extinction values, the efficiency of λ3130 and λ3660A is remarkably low. At λ3130A the efficiency is about one-eighth that at λ2537, λ2654 and λ3025A. At λ3660A it is exceedingly small, as even after a dosage of 74,500 ergs/mm.^{2} the eggs showed such slight indications of retardation that accurate measurement of the delay in cleavage could not be made.

Discussion

The failure to obtain acceleration by irradiation with either the lethal region in dosages below the retarding dose or by the non-lethal region of the quartz ultra-violet indicates that either (1) this part of the spectrum is ineffective for acceleration, (2) the dosage range was not the proper one, (3) sea urchin eggs are unfavorable material. It is possible that the sea-urchin eggs are already cleaving at a maximal rate and so the normal cleavage rate cannot be significantly accelerated at a given temperature.

The low retarding efficiency of the long wave-lengths of ultra-violet, 3130 and 3660A, is not surprising on the basis of previous investigations (Gates, 1929; Oster, 1934; Giese and Leighton, 1935*a*) which have indicated a very low efficiency of λ3130A in destroying cells. Wyckoff (1932) has shown that 47 times as much energy is necessary to kill 50 per cent of the bacteria at λ3132A as at λ2654A. The λ3660A is generally considered beyond the region of destructive action. On the other hand, Coblentz and Fulton (1924) consider this region in very high dosages bactericidal, and Hertel (1905) maintained that even at λ4400, 5230 and 5580A retardation of division of sea urchin eggs was obtained when the intensity was sufficiently great.

The high efficiency of λ2804A, whatever the absorption may be, is, however, surprising in view of the many investigations in which λ2654A has been found more effective as a bactericidal (Gates, 1929; Wyckoff, 1932; Ehrismann and Noethling, 1932; and Hollaender and Claus, 1936) and a photolethal agent (Oster, 1934, and Ehrismann and Noethling, 1932, on yeasts; Weinstein, 1930, on paramecia, and Mayer and Schreiber, 1934, on tissue culture). On the other hand, λ2800A has been observed to be more effective than λ2654A in a number of instances (Lassen, 1928; Sonne, 1929; and Giese and Leighton, 1935*b*, on paramecia and Ehrismann and Noethling, 1932, on *B. prodigiosus*).

Since λ2800 is near the middle of the strong absorption band (2700-2850A) of serum and egg albumin (Coulter, Stone and Kabat, 1936) while λ2654 is near the midpoint of strong absorption by thymus nucleic acid and some nucleoprotein derivatives (Heyroth and Loofbourow, 1933) a decision between the two is of importance to the theory of the mode of action of the radiation. Gates (1929) and others following him have postulated that since maximum efficiency in lethal action on bacteria is in the vicinity of λ2654A and since this is the seat of the maximal absorption by nucleoproteins the lethal effect is mediated by absorption of the radiations by the sensitive nucleoproteins. For retardation of the eggs, however, it seems as if the region of maximal effi-

ciency is that absorbed by the proteins of the cell other than nucleoproteins. This suggests that retardation of cleavage may be brought about not so much by an effect upon the nucleus as by interference with some part of the cleavage mechanism. The relative inefficiency of λ2537A which is absorbed strongly by the surface would tend to exclude the surface as the only or the major seat of action. Just which part is affected remains for further experimentation to discover.

Summary

1. The effects of a series of dosages of known intensities of monochromatic quartz ultra-violet light upon fertilized eggs of the sea urchin *Strongylocentrotus purpuratus* were studied.

2. Even at the highest dosages of λ3660A used the eggs cleaved at just about the natural rate of controls; λ3130A produced more pronounced retardation of cleavage; λ2537, λ2654 and λ3025A were about equally effective, and λ2804A most effective of all.

3. No signs of definite and marked acceleration were observed at any of the wave-lengths for any of the dosages employed.

4. The destructive effect may be mediated by some substance most sensitive to λ2804A.

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MONOCHROMATIC ULTRA-VIOLET RADIATION AS AN ACTIVATING AGENT FOR THE EGGS OF ARBACIA PUNCTULATA

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It has been known since 1914 that *Arbacia* eggs can be activated by the radiation from a quartz mercury vapor lamp. Loeb (1914) reported membrane elevation in unfertilized sea-urchin eggs. He obtained cleavage in these same eggs by a subsequent treatment with hypertonic salt solution. The occasional production of parthenogenesis by ultra-violet radiation alone has been reported by Lillie and Baskervill (1922), Tschahotine (1921) and others.

We observed during the summer of 1936 that the radiation from a very intense mercury vapor lamp would activate readily the unfertilized eggs of *Arbacia punctulata*. During the summer of 1937 we have been able to obtain activation in *Arbacia* eggs with monochromatic radiation and found that certain wave-lengths in the ultra-violet region are highly effective in producing parthenogenesis.

Experimental Technique

Experiment with Polychromatic Radiation

An intense beam of radiation from a vertical water-cooled, high pressure quartz capillary mercury vapor lamp (Daniels and Heidt, 1932; Hollaender and Stauffer, 1933) was made to cover by means of a right angle quartz reflecting prism and a quartz condensing lens a horizontal area of 2 by 18 mm. *Arbacia* eggs freshly removed from the ovaries and washed were covered to the height of about 3 mm. with sea water in Syracuse watchglasses, and moved through the beam in such a manner that the dishes were exposed for about 1 to 10 seconds, each egg thus receiving only a fraction of a second of exposure. The eggs in dishes which had received the appropriate quantities of radiation (3 to 5 seconds/dish) showed up to 90 per cent cleavage 3½ to 5 hours after exposure. The activation was not interfered with when the infra-red radiation was removed by means of a water filter. The removal of the ultra-violet radiation below 3000 A by means of an appropriate glass filter inhibited all activation. This definitely located the region in that part of the spectrum between 2000 and 3000 A.

Experiments with Monochromatic Radiation

The next step was to employ monochromatic radiation. We used for this purpose a technique similar to one described previously (Hollaender and Claus, 1936), with certain modifications. A diagram of the experimental arrangement is given in Fig. 1. *A* is the light source (water-cooled high pressure quartz capillary lamp, loc.) using the current from a 500 volt d.c. supply, burning on about 350 volts and 3.5 amperes.[1] *C* are quartz condensing lenses. *D* are quartz prisms mounted on an adjustable turn-table. *E* is a right angle quartz reflection prism mounted in such a manner that it can be turned through an

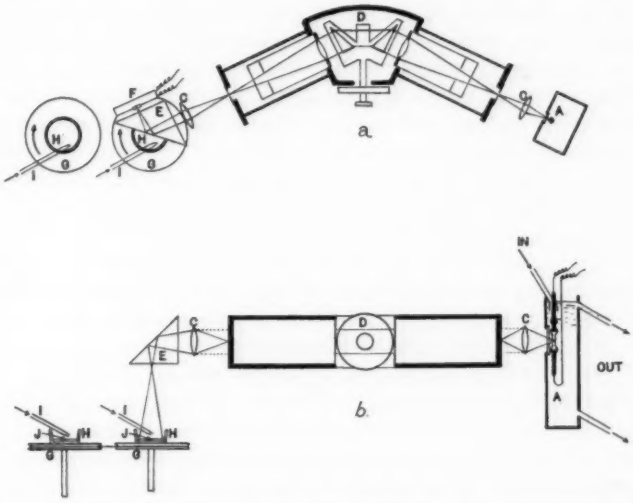

Fig. 1. Diagram of experimental arrangement for the exposure of *Arbacia* eggs with monochromatic radiation. *a*, horizontal, and *b*, vertical cross section. For details see text.

angle of 90°. This makes it possible for the reflected beam to cover a horizontal area of the exposure dish or the vertical receiving surface of the thermopile. *F* is a vacuum thermopile in casing with quartz window connected with a high sensitivity galvanometer (not shown in diagram). The sensitivity of the thermopile was determined against a Bureau of Standards "standard lamp." *G* are two platforms rotated by a small motor (not shown) at identical slow speed. *H* are small

[1] Special thanks should be given here to Mr. Boss and Dr. Pond of the Marine Biological Laboratory who made available a rectifier-transformer delivering the necessary high voltage.

(33 mm. diameter) Stender dishes each fastened to the center of a platform. *I* are the tapered outlets which direct a slight stream of air against the surfaces of the liquids in *H*.

For each experiment, the eggs of one *Arbacia* were washed, and about 300 to 600 eggs placed in each Stender dish, and covered to the height of about 3 mm. with sea water. The dish to be exposed was fastened to the platform under the reflection prism, the control dish was fastened to the platform to the left. The two platforms (*G*) with the dishes (*H*) were made to rotate at a uniform speed. Slight air streams of the same pressure for both dishes were directed against the outer quarters of the water surfaces. The rotation of each dish had a tendency to move the eggs against the outer rim, and the air stream tended to bring the eggs to the center of the dish. These two opposed forces made the eggs roll in the dish in such a manner that each egg occupied every possible position in the dish many times during the course of exposure, and every section of the surface of each egg received equal quantities of the radiation.

The efficiency of the stirring was checked in the following manner: Uranium glass particles were placed in the dish with sea water, the dish stirred as described above, and the beam of ultra-violet permitted to fall on the dish. The fluorescing moving glass particles illustrated the efficiency of stirring. If this method of stirring is used carefully, slow rotation, not too strong a beam of air, no damage will be done to the eggs. These precautions were necessary since the beam of monochromatic radiation covered only one-fifteenth of the bottom of the exposure dish. No other mechanical stirring device was found which would fulfill the condition outlined without damage to the eggs.

Before the actual exposure was started, the reflection prism was turned 90° so that the beam from the monochromator would fall on the receiving surface of the thermopile (*F*) as shown in the diagram of the horizontal cross section of the apparatus (*a*). After the energy reading was taken, the prism was turned through 90° and the beam made to cover the described area of the exposure dish as shown in the vertical cross section (*b*), having the dishes rotate during the entire time. Most exposures were for 3 to 10 minutes, but exposures as short as 30 seconds or as long as 30 minutes were made occasionally. Energy readings were taken before and after each exposure. If the exposure was longer than 15 minutes, an additional reading was taken during the middle of the exposure.

The effect of radiation of the following wave-lengths was tested: 2260, 2300, 2380, 2480, 2537, 2650, 2805, 2950, 3050, 3150, 3350, and 3650 A. Since activation was produced with whole eggs only by wave-lengths below 2805 A, and especially with radiation of 2260 to 2380,

most of the work described concerns itself with these latter wave-lengths.

After irradiation the eggs were removed to larger dishes and covered with more sea water. Usually the eggs were divided into several lots. One lot kept at room temperature for 4 to 5 hours and then 2 drops of 2 per cent formaldehyde per dish added, and the number of cleaved and uncleaved eggs determined. Another lot kept in the refrigerator at about 10° C. for 15 to 20 hours and then the percentage of cleaved eggs determined. It was necessary to keep the eggs at two different temperatures since the laboratory temperature during the summer of 1937 would rise often to 27°, a temperature critical for the development of the eggs. Apparently the cold seemed to be beneficial.

It is known that *Arbacia* eggs can be activated by many chemicals and by certain physical treatments beside radiation. Special care was taken to recognize such effects if they were produced inadvertently, in these experiments, and to avoid the error of interpreting them as radiation effects. For this reason each group of experiments had the following controls:

1. Eggs of each sea urchin used were fertilized with fresh sperm and only those eggs used which when tested gave a high percentage of cleavage.

2. Each group of four to five exposures had one standard control. That is, these control eggs were handled in an identical manner with the exposed eggs with the exception that they did not receive any ultra-violet radiation. Controls included in the lots were kept at room temperature as well as at 10° C. These controls gave a check in regard to mechanical as well as temperature effects.

3. In the course of this investigation, controls of the following type were used, one to each group of experiments. Dishes with the usual quantities of sea water but without eggs were irradiated with the different wave-lengths and given the different times of exposure found effective in the regular tests. After radiation, unfertilized eggs were added to the irradiated water and permitted to stand with the dishes of exposed eggs. With the wave-lengths and the times of exposures used in this investigation, no effect by irradiated sea water on the eggs could be recognized. If the time of exposure was extended for many hours, effects of the radiation on sea water were found (Rakestraw and Hollaender, 1936). But this lies outside the subject of this paper.

Results

The results of a typical experiment are given in Table I. The manner of calculating the incident energy/egg is illustrated in the following protocol.

TABLE I

Dish	Time of exposure	Incident energy/egg	Log of incident energy	Eggs cleaved : not cleaved	Percentage cleaved
83	30 seconds	0.335	0.525 −1	3 : 100	3
79	60 seconds	0.67	0.826 −1	68 : 220	23.8
77	120 seconds	1.37	0.137	107 : 226	32.1
75	240 seconds	2.67	0.426	283 : 73	79.5
73	360 seconds	4.0	0.602	316 : 62	83.5
82	0 (control)	0	0	0 : 252	0
76	240 seconds	2.67	0.426	268 : 31	89.5
78	120 seconds	1.37	0.137	241 : 81	75
79	0 (control)	0	0	0 : 212	0

Protocol of Part 2 of Experiment 28 August 8, 1937. Eggs removed at 10 A.M. washed twice with sea water, some fertilized, cleaved normally. Kept dish in sea water sink until eggs were needed. Standard exposure dish used. Exposed to 2260 A, 37.5 cm. galvanometer deflection, 61.3 ergs/cm. deflection. The beam covered $2 \times 28 = 56$ mm.^{2}, diameter of dish 33 mm., area $= .7854 \times 33^2 = 855$ mm^2. Part of area of the dish exposed $\frac{56}{855} = 15.3^{-1}$. Formula for calculation of incident energy received per egg with diameter of 74μ.

$$\frac{E \times T \times C \times f}{a} = I$$

E = Incident energy in ergs/second $= 37.5 \times 61.3$
T = Time of exposure = 30 seconds
C = Cross-sectional area of the egg $4300.8\mu^2$
a = Area of beam $= 56 \times 10^6\mu^2$
f = Fraction of time each egg exposed $= \frac{1}{15.3}$
I = Incident energy/egg

$$\frac{37.5 \times 61.3 \times 30 \times 4300.8}{56 \times 10^6 \times 15.3} = 0.348 \text{ ergs/egg}$$

The eggs kept in the first six dishes as given in Table I were held at room temperature until 4 P.M. and then formaldehyde added. Duplicate set of dishes kept in refrigerator for 20 hours. Results of 3 dishes kept in refrigerator are given. Eggs in rest of dishes kept in refrigerator used for cytological examination.

Figure 2 illustrates the results of a typical experiment if one wavelength only was investigated, permitting the determination of many points on the curve. One curve is for eggs kept at room temperature

and the other curve for those kept at 10° C. It would appear that the eggs in the dishes kept at room temperatures were given the formaldehyde which stopped their development too early. This was unavoidable, since it was not always possible to determine in advance best time of cleavage, and waiting too long at room temperature brought about occasionally cytolysis of the eggs.

There was a considerable amount of variation in the eggs of different females in their sensitivity to radiation. Thus the incident energy of activation at wave-length 2260 A varied between 2.25 to 4.2 ergs per egg for 80 per cent activation. For this reason the activation energy at different wave-lengths was always determined for the eggs from one

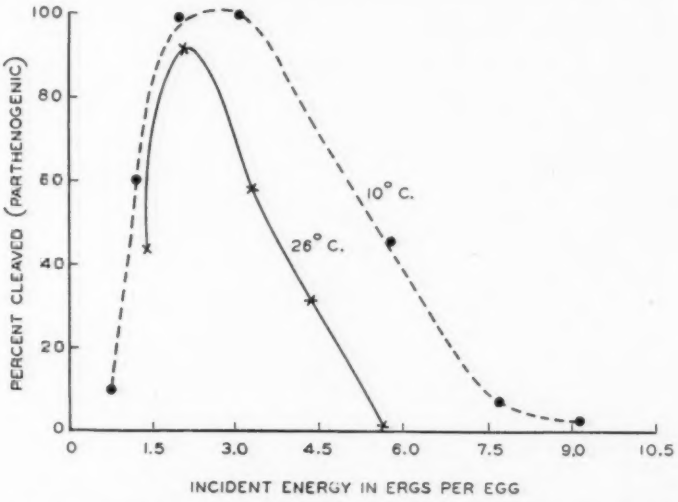

FIG. 2. Graphs showing the percentage of unfertilized eggs cleaved and the incident energy per egg. One set of eggs was kept at 26° C. and one at 10° C. after exposure.

individual and in no case has the relative energy necessary to produce activation in its dependence on wave-length been any different from that given in Fig. 3. That is, the lowest energy necessary to produce activation was at 2260 A and activation became negligible around 2500 A, even with relatively high energy values. Figure 3 is not as complete as is desirable because the number of exposures that can be handled in a certain time is limited. Complete curves as given in Fig. 2, if obtained for many wave-lengths, require more time than would be safe to keep eggs from one individual.

The irradiated eggs showed normal fertilization membranes. But

only eggs which showed at least the two-cell stages were called activated. This took usually three to five hours at room temperature. Many of the activated eggs went to further stages of development. Sixteen and 32-cell stages were observed, but some of the eggs cytolyzed after two or three divisions. It was necessary to limit this part of the investigation to the observation of divisions which gave the most reliable counts, i.e., the two-cell stage.

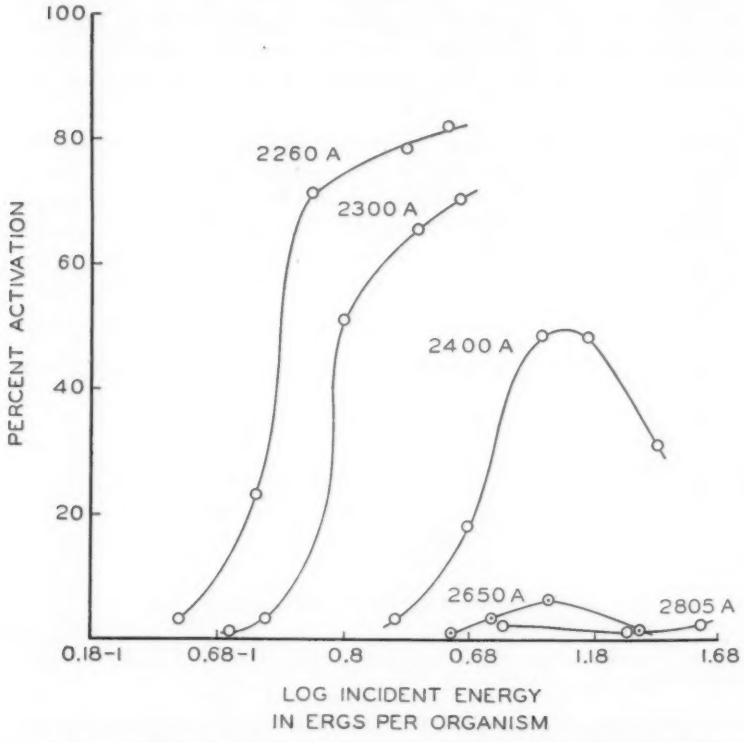

FIG. 3. Graphs showing percentage of the unfertilized eggs activated by monochromatic ultra-violet radiation against the log of the incident energy at five wave-lengths.

Figure 4 illustrates graphically the effectivity of the different wavelengths in producing activation of *Arbacia* eggs, taking the energy necessary at 2260 A as 100. The absorption spectrum of an egg of *Paracentrotus lividus* as determined by Vlès and Gex (1927) is given. Also, the inactivation spectrum of the virus of typical tobacco mosaic

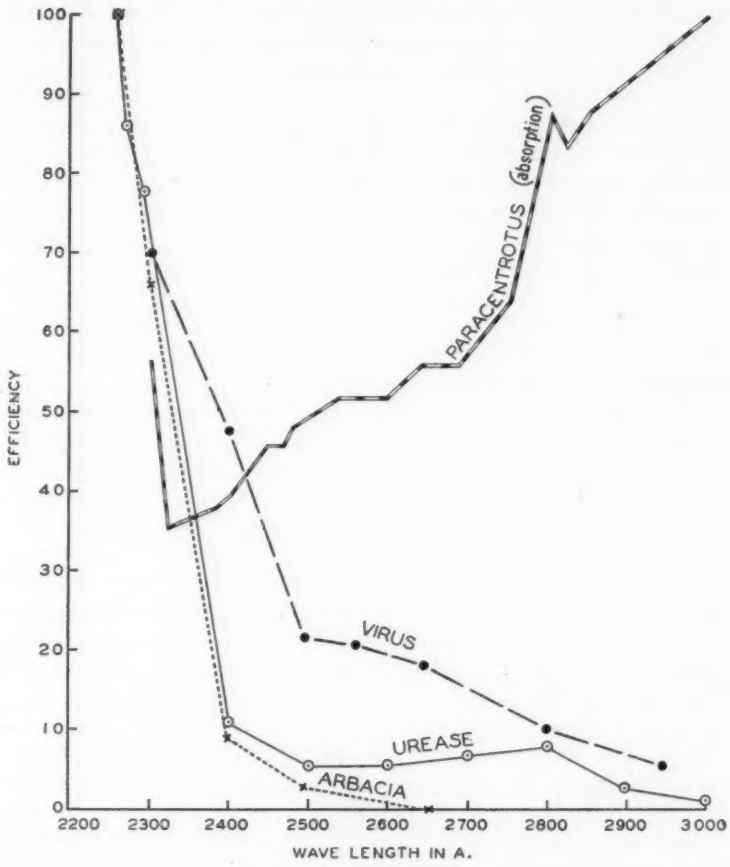

FIG. 4. Summary graph giving (1) the efficiency of the different wave-lengths in producing 50 per cent activation in *Arbacia* eggs; (2) The destruction curve of urease as described by Kubowitz and Haas (1933); (3) the destruction curve of the virus of typical tobacco mosaic (Hollaender and Duggar, 1936); and (4) the absorption curve of one egg of *Paracentrotus lividus* as given by Vlès and Gex (1928).

The data for the relative wave-length dependence of the action of monochromatic ultra-violet radiation on these different agents have been recalculated from the originals to make a comparison possible.

(Hollaender and Duggar, 1936) and the inactivation spectrum of urease (Kubowitz and Haas, 1933—see also Gates, 1934) are given. Data from these different sources have been recalculated to fit into the scheme of this graph and are given only for comparison, i.e., to show

the efficiency of different wave-lengths to bring about the investigated effects, and should not be compared for absolute energy values.

The absorption spectrum of an egg of *Paracentrotus lividus* as given by Vlès and Gex is not too helpful as a clue in interpreting the activation (parthenogenesis) spectrum, since it is that of a different species of sea urchin. The taking of such absorption spectrum is difficult and it is felt that it would be very worth while to repeat this work using a somewhat different technique from that used by Vlès and Gex. Coloring matter and other highly absorbing materials contained in the eggs, which vary for different species, mask probably to a large degree the absorption of the essential parts of the egg. However, the rapid increase of the absorption at wave-lengths between 2325 and 2300 (the lowest wave-length tested by Vlès and Gex) indicates a possible correlation with the activating radiation.

The inactivation spectra of urease and a typical virus have been given since these curves follow closely the activation curve of *Arbacia*. The virus curve has been suggested (Hollaender and Duggar, 1936) as the inactivation curve of a protein. The isolation of a crystalline protein with high virus activity from a typical tobacco mosaic virus suspension has been described by Stanley (1937). All these compounds, i.e., proteins, show their highest sensitivity in the ultra-violet below 2400 A. Nucleic acids have their most pronounced absorption around 2650 A (see also Caspersson, 1937) a wave-length not effective in producing activation of *Arbacia* eggs. It would appear from this that the possibility of the eggs being activated by a mechanism which starts with the absorption of the ultra-violet by nucleic acids is excluded. But not too definite conclusions as to the type of compound can be made from the high sensitivity of the *Arbacia* at the short ultra-violet, since besides proteins many other types of organic compounds also increase absorption at these short wave-lengths. We know very little of the effect of monochromatic ultra-violet radiation on proteins, especially the effect of energies which have been used in these tests.

Cytological observations in connection with this work are given in a separate paper (Harvey and Hollaender, 1938).

I wish to thank Dr. E. B. Harvey for her coöperation throughout the course of these experiments and for her criticism of this paper, the University of Wisconsin for placing laboratory space at the Marine Biological Laboratory at our disposal, and the Radiation Committee, National Research Council, for the supply of equipment.

SUMMARY

1. A method for the effective irradiation of *Arbacia* eggs with measured quantities of monochromatic ultra-violet radiation is described.

2. The activation (parthenogenesis) of *Arbacia* eggs by polychromatic and monochromatic radiation is demonstrated.

3. The energy requirements and wave-length dependence of activation have been worked out. The wave-lengths most effective are in the shorter ultra-violet.

4. The wave-length dependence of the activation curve of *Arbacia* is compared with the inactivation curves for urease and the virus of typical tobacco mosaic.

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PARTHENOGENETIC DEVELOPMENT OF THE EGGS AND EGG FRACTIONS OF ARBACIA PUNCTULATA CAUSED BY MONOCHROMATIC ULTRA-VIOLET RADIATION

ETHEL BROWNE HARVEY AND ALEXANDER HOLLAENDER

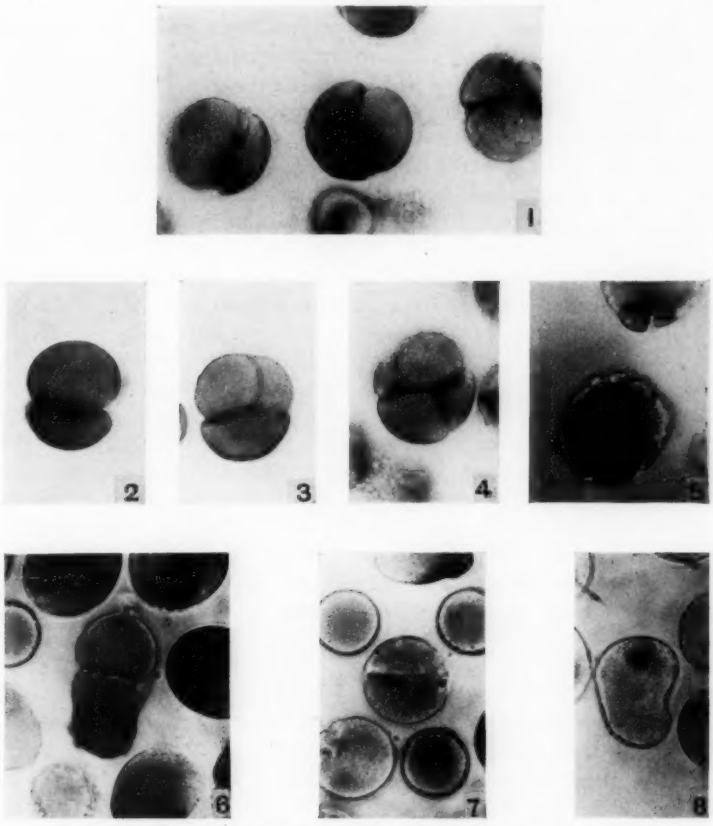
(From the Marine Biological Laboratory, Woods Hole, Mass., the Biological Laboratory, Princeton University and the Washington Biophysical Institute)

Normal unfertilized (uncentrifuged) eggs of *Arbacia punctulata* can be activated by ultra-violet radiation, either by the full arc for 1/10 to 10 seconds or by monochromatic radiation of 2260–2480A for two to eight minutes. The method has been fully described in the preceding paper in this journal (Hollaender). The eggs throw off a normal fertilization membrane and pass through several cleavages (Photographs 1–5). The cleavages are much delayed in comparison with normal fertilized eggs[1] just as they are after treatment with other parthenogenetic agents, such as hypertonic sea water. In some experiments, with the optimum treatment, all the eggs are activated and after five hours, 80 per cent have cleaved (Photograph 1); frequently the first cleavage gives two unequal cells (Photograph 2). In many eggs a second cleavage follows in an hour; it may be asynchronous in the two blastomeres resulting in a 3-cell stage (Photograph 3), or synchronous giving a typical 4-cell stage (Photograph 4). Further cleavages result in 8 to 16-cell stages (Photograph 5), but no further development was observed.

Whole eggs which have become stratified and elongated by a strong centrifugal force (10,000 $\times$ *g* for 3 minutes) are activated by the same radiation as the uncentrifuged eggs; they form fertilization membranes following the irregular contour of the egg, just as similar fertilized eggs do, or as do those artificially activated by hypertonic sea water. About 50 per cent of the eggs cleave, in about the same time as the uncentrifuged—that is, after four to five hours. The first cleavage plane usually comes across the short axis of the elongate egg (Photograph 6) just as it does in fertilized or other parthenogenetic eggs of the same shape (E. B. Harvey, 1932, 1936). When the centrifuged egg is spherical or nearly so at the time of radiation, the cleavage plane tends to come in through the equator, just as in normal fertilized eggs. The centrifuged eggs which have been radiated pass

[1] First cleavage of the normal fertilized egg takes place in 50 minutes at 23°.

PLATE I



Photographs of living *nucleate* eggs, taken with a Leica camera and water immersion lens.

1. Unfertilized uncentrifuged eggs, six hours after radiation of 2380A for five minutes. All eggs in field cleaved.

2. Same, 2-cell stage unequal. Cf. Photographs 20 (red half) and 26 (yolk quarter).

3. Same, seven hours after radiation. Three-cell stage. Cf. Photographs 21 (red half) and 27 (yolk quarter).

4. Same, 4-cell stage. (Cf. Photograph 28.)

5. Same, eight hours after radiation. Sixteen-cell stage. Cf. Photograph 23 (red half).

6. Centrifuged whole egg, six hours after radiation with full arc five seconds. Two-cell stage. Cf. Photographs 30, 31 and 36 of E. B. Harvey, 1936.

7. White half-egg, same treatment. Two-cell stage.

8. Another white half-egg, five hours after radiation. Nuclear membrane has just broken and egg is somewhat amoeboid.

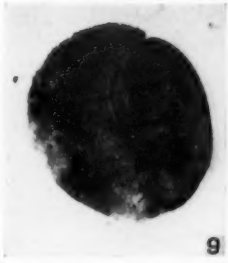
through several cleavages, but stop at about the same stage as the uncentrifuged.

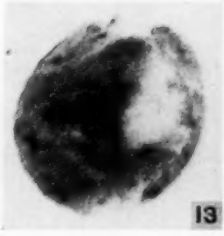
The nuclear changes leading up to first cleavage can be observed in the living egg, especially in the centrifuged egg. After radiation, the usual nuclear changes take place, the enlargement of the nucleus, breaking of the nuclear membrane and the formation of the amphiaster; it is a perfectly normal cleavage in many observed cases. Prepared sections of later stages (8 hours after activation) of radiated uncentrifuged eggs showed some normal prophase, metaphase and anaphase figures (Photographs 9, 10 and 11). Typically there are no asters at the poles of these spindles (Photographs 10, 11) but occasionally they are well-formed (Photograph 9); and large asters may occur independently of chromosomes, in the cytoplasm (Photograph 17). Spindles without asters are characteristic of somewhat later cleavages of the normal fertilized *Arbacia* egg when the cells are very small, but in the early stages corresponding to those pictured here, the asters of the normal dividing egg are very conspicuous. Photograph 12 shows the quite normal-looking nuclei resulting from the first division of a radiated egg. Together with the fairly normal mitotic figures, and in much greater abundance, are abnormal figures. The most characteristic feature of the radiated eggs is the occurrence of multipolar spindles, each spindle with its chromosome plate. The axes of the spindles may be parallel (Photographs 13, 14) or they may lie at any angle with regard to each other, forming a rather confused figure (Photographs 15, 16). The chromosome number appears to be sometimes haploid and sometimes diploid in the more simple type of spindle, but is very large in the compound spindles, and judging from the formation of these spindles should be, at least in some cases, tetraploid (Photograph 14).

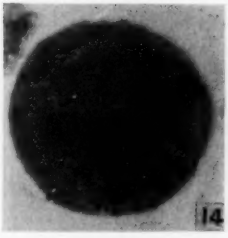
White half-eggs, obtained by breaking the whole eggs into two parts by centrifugal force,[2] are also activated by ultra-violet radiation. These are the lighter halves and contain the female nucleus. When activated, they form fertilization membranes, and usually after five hours cleave equally since they are spherical (Photograph 7), just as do white halves which are fertilized. Subsequent cleavages have given 8-cell stages, but no further development. These white half-eggs are much more sensitive to the same amount of ultra-violet radiation than are the whole eggs, normal or centrifuged. With the optimum treatment, only 5 per cent of the eggs had cleaved after five hours; those radiated slightly longer were all cytolyzed after five hours, and those radiated slightly less were not activated. The

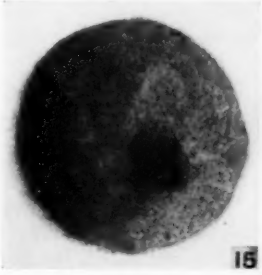
[2] For a description of the method, see E. B. Harvey, 1932 and 1936.

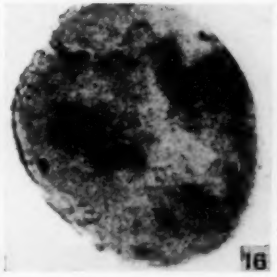
PLATE II











Photographs of prepared and stained sections of whole uncentrifuged eggs, taken with Leica camera and oil immersion lens. The eggs were fixed seven hours after radiation with full arc for five seconds.

9. Normal prophase with asters.
10. Normal metaphase, but no asters.
11. Normal anaphase, but no asters.
12. Two-cell stage, with normal nuclei.
13. Beginning anaphase, several spindles combined, in same plane; side view.
14. Metaphase plates of four spindles combined in same plane.
15. Metaphase, several spindles combined in various planes.
16. Same, polar view.
17. Large cytasters without spindles or chromosomes.

"dose" is rather critical. There is a tendency for the white half-egg to become aspherical and amoeboid (Photograph 8).

Red half-eggs, the heavier halves obtained by breaking the whole egg into two parts by centrifugal force, do not contain a nucleus, nor any part of it; the nucleus goes invariably into the lighter white halves. The red half-eggs are, nevertheless, activated by ultra-violet radiation. They are activated by the same radiation as the whole eggs and also by a band of longer wave-length, 2650–3300A for four to twelve minutes, a treatment which does not affect the whole eggs and white halves, but does affect the yolk and pigment quarters. They form a fertilization membrane and a thick ectoplasmic layer (Photograph 18). After an hour and a half, a clear area is observed in the less dense portion, a sort of pool, just as in the red halves activated by hypertonic solutions (E. B. Harvey, 1936, p. 111). In a few cases, large monasters in this area are observed four hours after radiation. These have also been found in prepared sections of the red half-eggs (Photograph 31). A notch frequently appears at the equator of the more spherical red halves indicating the beginning of a cleavage plane (Photograph 19). Two-cell stages of these partheno-

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PLATE III

Photographs of living *non-nucleate* eggs taken with Leica camera and water immersion lens, except the last photograph, which is from a prepared slide taken with an oil immersion lens.

18. Red half-egg, six hours after radiation of 2380A for five minutes. Note fertilization membrane and ectoplasmic layer.

19. Red half-egg, seven hours after radiation, full arc 10 seconds. Indentation at equator.

20. Red half-egg, eight hours after radiation, full arc five seconds. Two-cell stage. (Cf. Photographs 1 and 2 of similar *nucleate* egg.)

21. Red half-egg, seven hours after radiation of 2380A for six minutes. Three-cell. (Cf. Photograph 3 of similar nucleate egg.)

22. Same lot. Four-cell.

23. Red half-egg nine hours after radiation of 2380A for six minutes. Eight to twelve-cell. (Cf. Photograph 5 of similar nucleate egg.)

24. Red half-egg seven hours after radiation of full arc five seconds. Amoeboid.

25. Same egg one-half hour later.

26. Yolk quarter seven hours after radiation of 3300A for five minutes. Two-cell. (Cf. Photographs 1 and 2 of nucleate egg.)

27. Same egg one hour later. (Cf. Photograph 3.)

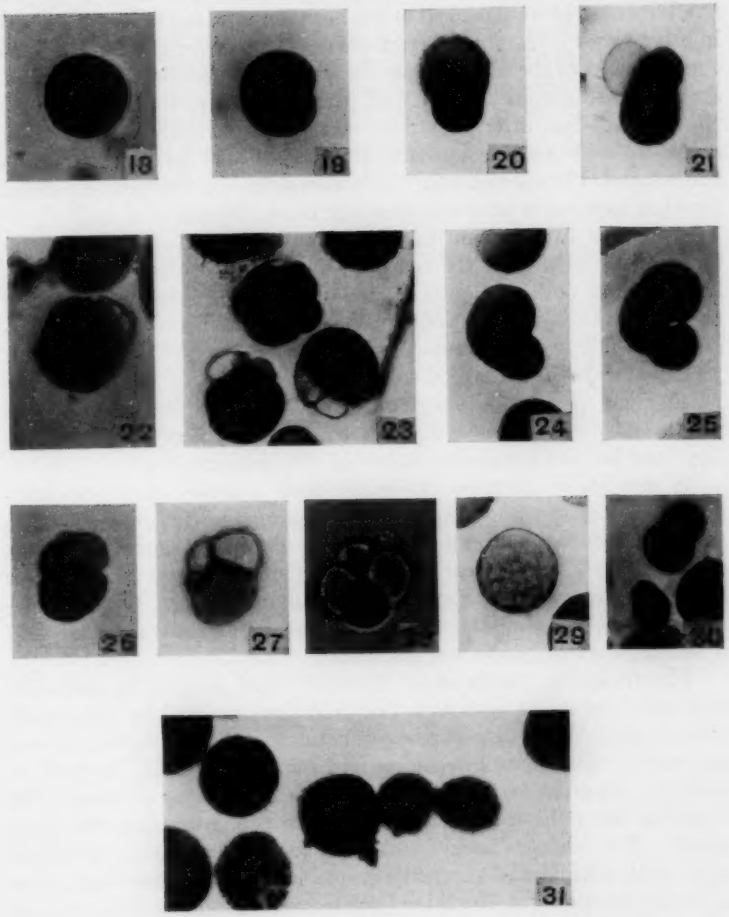
28. Yolk quarter eleven hours after radiation of 2380A for six minutes. Four-cell. (Cf. Photograph 4.)

29. Yolk quarter seven hours after radiation of 3300A for nine minutes. Multi-astral.

30. Pigment quarter (lower cell) seven hours after radiation of full arc five seconds. Amoeboid.

31. Prepared and stained section of red half-eggs seven hours after radiation with full arc for five seconds. Note large aster in center cell and absence of chromosomes and spindles. (Cf. similar nucleate cells of Plate II.)

PLATE III



genetic merogones occur (Photograph 20) and a further cleavage gives a 3- and 4-cell stage (Photographs 21, 22). A 12-cell stage is the latest that has been observed (Photograph 23). Later on, however, some of these eggs are found with many asters, a possible precursor to a blastula and similar to what has been found in red halves activated in other ways. The similarity of the development of the non-nucleate egg with that of the nucleate may be seen by comparing similar stages, such as Photographs 21 with 3, 23 with 5 and 28 with 4. Frequently the red half-egg, after radiation, becomes aspherical and somewhat amoeboid with a tendency to form and split off a lobe from the pigmented end (Photograph 24). This is sometimes completely separated by what appears to be a true cleavage plane (Photograph 25).

The yolk quarter and pigment quarter which are obtained by further centrifuging of the red half, and which are, of course, non-nucleate, are both activated by radiation of the same wave-lengths as the red halves. The yolk quarters cleave several times (Photographs 26–28) to give as many as eight or twelve cells. Multi-astral yolk quarters also occur (Photograph 29).

Pigment quarters, although only one-fourteenth the volume of the whole egg, show evidence of activation in that they form an ectoplasmic layer and become somewhat amoeboid (Photograph 30).

A few clear quarters (nucleate) and granular quarters (non-nucleate) obtained by further centrifuging of the white half-eggs also show signs of activation by radiation. The clear quarters become amoeboid and the nucleus breaks down, and the granular quarter forms a fertilization membrane and ectoplasmic layer. Owing to the difficulty in obtaining these fractions, only a few were radiated, and a thorough investigation was not made.

The foregoing results show definitely that ultra-violet radiation acts upon the whole eggs and their fractions obtained by centrifugal force exactly like other parthenogenetic agents such as hypertonic sea water. The most interesting and important result, however, is that the radiation activates the ***non-nucleate*** fractions of eggs. The action of the radiation must, therefore, be primarily upon the cytoplasm. The nuclear changes leading up to first cleavage, in the whole eggs, both normal and centrifuged, and in the white half-eggs, seem perfectly normal, but the later spindles and chromosome groups are often quite irregular and abnormal. The conclusion is drawn, then, that the ultra-violet radiation affects primarily the cytoplasm, but there is also an effect upon the nucleus. Whether this is a direct action of the radiation on the nucleus or a secondary effect due to changes first produced in the cytoplasm by the radiation, it is difficult

to say. It would rather seem that the effect on the nucleus is secondary since the nuclear phenomena accompanying the *first* cleavage appear to be unaffected.

Summary

1. Ultra-violet radiation either by the full arc for 1/10 to 10 seconds or by monochromatic radiation of 2260–2480A for two to eight minutes causes activation in normal unfertilized eggs of *Arbacia punctulata*, in the centrifuged whole eggs and in the halves and quarters derived from the whole eggs by centrifugal force.

2. Cleavages of the eggs and fractions have been obtained up to a 12- to 16-cell stage.

3. The nuclear phenomena of the nucleate eggs seem to be normal for the first cleavage, but in later cleavages many abnormal mitoses occur, chiefly multipolar and anastral.

4. Fractions of eggs without nuclei are activated by ultra-violet radiation just as they are by hypertonic sea water. These parthenogenetic merogones cleave and go to a 12-cell stage quite similar to that of a nucleate egg.

5. The ultra-violet radiation affects, therefore, primarily the cytoplasm.

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STUDIES IN THE PIGMENTARY SYSTEM OF CRUSTACEA

III. Light-intensity and the Position of the Distal Retinal Pigment in Leander adspersus

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(From the Biological Laboratories, Harvard University, and the Department of Zoology, Oxford University)

Introduction

While studying retinal pigment migration in a Mediterranean decapod, *Leander adspersus*, we noticed that the position of the distal pigment cells in the eyes of an animal kept on an illuminated black background was different from that in a shrimp maintained on a similarly-illuminated white background. Although it has been previously shown that the light-adapted condition of the distal retinal pigment in crustaceans is effected by a hormone having its origin in the eye-stalks (Kleinholz, 1936), it has been assumed that the position of the distal retinal pigment at any one time was to be found in either the light-adapted or the dark-adapted condition.

The preliminary observation mentioned above, if verified by further study, would indicate that the position of the retinal pigment could be graded between the limits of dark- and of light-adaptation, and that the gradation could be achieved by varying not only the intensity of light but also the color of the background. This suggests, furthermore, that the pigmentary reactions of the crustacean retina are not entirely determined by the intensity of incident light, but are possibly regulated to some extent by the intensity of reflected light, or by some relation between these two factors. Such a possibility is supported by the fact that Keeble and Gamble (1904), Brown (1936), and Sumner and Doudoroff (1937) have found the chromatic reactions of crustaceans and fishes to be determined by a similar relationship (the intensity of incident light/the intensity of reflected light).

We therefore decided to investigate the relation between light intensity and the position of the distal retinal pigment in the shrimp, *Leander adspersus*.

We are indebted to Professor R. Dohrn for the facilities granted us at the Stazione Zoologica in Naples. One of us (L.H.K.) takes this

[1] Sheldon Fellow, Harvard University.

[2] Naples Biological Scholar, Oxford University.

opportunity to thank the Committee for permission to occupy the Jacques Loeb Memorial Table.

Materials and Methods

To reduce the error introduced by variations in the size of the eye among different individuals, only those shrimps measuring exactly 1.8 cm. from the tip of the rostrum to the posterior margin of the cephalothorax were used in these experiments. Two glass vessels, each about 10 inches in diameter and 5 inches deep, were prepared for the black and the gray backgrounds. The inside of one was covered with a melted mixture of paraffin-wax and lampblack; when this cooled it gave a uniform dull-black background. The gray background was prepared by first applying the black paraffin and then lining the inside of the dish with white-waxed tissue paper.

Ordinary electric-light bulbs of 25, 40, 50 and 100 watts were used as sources of illumination. In most of the series the bulbs were kept at a distance of 22 inches from the bottoms of the containers, but in two cases (series *B*-3 and *A*-4) the position of the electric lamp was adjusted to give ratios which were desired as points on the scale. Exact quantitative measurements of light-intensity were not possible because of the lack of an appropriate photometer. Since only the ratios of incident to reflected light were sought, these were obtained quantitatively in a manner adequate for the purpose of these experiments by using a "Sixtus" photometer (Leica-model). The scale of this meter which expresses light-intensity in terms of seconds necessary for photographic exposure (the lower the numerical value of the reading the higher the intensity of light) gave relative values for the various intensities and backgrounds. The intensity of incident light was measured at the level of the bottom of the vessel. Each value for the intensity of reflected light was the average of 10 readings taken against the bottom of the empty container.

At the start of an experiment, sea water was poured into the dish to a level of 2.5 cm., 5 measured shrimps were introduced, and the temperature of the water taken. Twenty-four hours were allowed for retinal adaptation. At the end of 12 hours the temperature of the water was again taken and the water changed. In no case had the temperature risen more than 1.6°C. at the end of any twelve-hour interval. After 24 hours the eyes were fixed.

The animals were immersed in hot water (80°C.) for 10 seconds to fix the position of the retinal pigments. The eyes were then excised and transferred to a modified Bouin's solution (containing 7 parts of glacial acetic acid) for 24 hours. They were next placed successively in 70 per cent alcohol for 3 hours, in dioxane for 12 hours and in paraffin-wax

for 3–5 hours. Before the final embedding the eyes were oriented in the melted paraffin with the dorsal side uppermost (readily recognized by the black pigment-spot located at the junction of the retinal and non-retinal portions of the eye-stalk). Serial sections were cut at 20μ and were mounted unstained. Measurements of the position of the distal retinal pigment were made with an ocular micrometer through the middle of the eye, from the basement membrane to the proximal end of the distal pigment cells in 10 serial sections which included the dorsal pigment spot (Fig. 3). The structure of the eye of *Leander* is similar to that of *Palaemonetes* (Kleinholz, 1936).

In one experiment (Series I) the animals were illuminated from below by a 40-watt lamp, at a distance of 22 inches from the bottom of the clear glass container. The dish used for gray background was inverted over the container.

Relation of Light-Intensity to Pigment Migration

The data from these experiments are arranged in Table I. Figure 1

Table I

Light-intensity and the position of the distal retinal pigment. Intensity of light was measured in relative rather than in absolute units (see under "Methods"). Series *A* were on the gray background, Series *B* on the black background, Series *D* in total darkness. *I*, intensity of incident light; *R*, intensity of reflected light; the ratio, *I*/*R*, was multiplied to give whole numbers; *P.P.*, distance of the proximal end of the distal pigment cells from the basement membrane in μ, and the average deviation within a series.

Bulb	Series	*I*	*R*	$\frac{I}{R} \times 1000$	*P.P.*
25	*A*-1	0.40	15.0	26	295μ ± 17μ
40	*A*-2	0.25	6.3	39	241μ ± 9μ
50	*A*-4	0.18	3.5	51	190μ ± 18μ
100	*A*-3	0.125	2.0	62	167μ ± 15μ
25	*B*-1	0.40	45.0	9	372μ ± 18μ
40	*B*-2	0.22	13.0	17	319μ ± 18μ
100	*B*-3	0.18	8.1	22	311μ ± 24μ
—	*D*	—	—	—	526μ ± 22μ

shows that, provided the background is kept constant, the amount of migration of the retinal pigment is directly proportional to the intensity of incident light (limited, however, by the total migration possible in passing from complete light- to complete dark-adaptation: about 360μ in the standard-size animals used here). The effect of backgrounds is shown by the different extents to which the distal pigment migrated in animals kept in the black and in the gray containers, but at the same intensities of incident light. The prolongations of the two curves meet

at a point — 515μ — where the positions of the distal pigment should be identical, regardless of the color of the background. Obviously, this theoretical position of the pigment should be found in the retinas of animals adapted to darkness. Measurement of the position of the retinal pigment in 7 dark-adapted eyes gave an average value of $526\mu \pm 22\mu$, in good agreement with the theoretical value.

When the data in the last two columns of Table I are plotted (Fig. 2), it becomes evident that, under the conditions of illumination used, the position of the distal pigment is in direct proportion to the ratio, $\frac{I}{R}$.

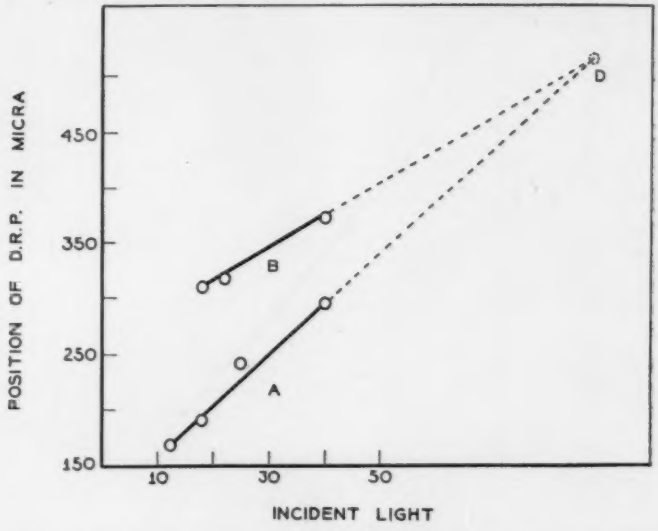

FIG. 1. The relation between intensity of incident light and position of the distal retinal pigment as measured from the basement membrane to the proximal end of the pigment cells. *A*, series on the gray background; *B*, series on the black background; *D*, theoretical position of the pigment in darkness (see text).

Whether the same direct relation would hold for the lower intensities of light, such as used by Brown (1936) in studying the chromatophoral behavior of a teleost, is not known.

Keeble and Gamble (1904, p. 354) found that color reactions were normal in crustaceans which were illuminated from below against white or black backgrounds, and that an implied dorsi-ventrality was therefore "not due to a permanently fixed structural difference in the two sides of the eye" but was produced "by some mobile structure which

takes up its position in response to the light conditions." [3] On examining sections of retinas in any particular stage of light-adaptation, it is seen (Figs. 4–7) that the pigment in the dorsal portion of the eye has always undergone a greater amount of migration (toward the basement membrane) than that in the ventral portion. But since migration of the distal pigment is under hormonal control (Kleinholz, 1936), the reason for the unequal position of this pigment is not clear. If the variation in position of the pigment of the dorsal and ventral regions of the retina is constant under all conditions of illumination, the inequality could be attributed to greater sensitivity of the pigment cells in the dorsal portion of the retina to the eye-stalk hormone. If, on

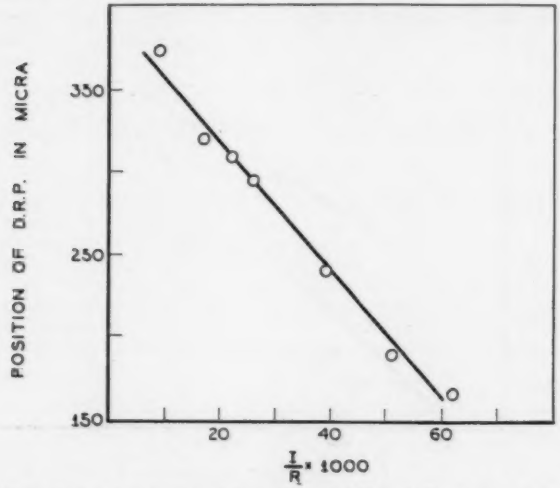

FIG. 2. The relation between position of the distal retinal pigment and the ratio, $\frac{\text{incident light}}{\text{reflected light}}$.

the other hand, this difference could be reversed by changing the method of illumination, the above explanation would not be valid; in such an event the greater amount of migration in the portion of the eye most strongly illuminated (toward the source of light) might be due to an additional direct effect of light on those cells. This point was

[3] Butcher and Adelmann (1937) reported, however, that illumination of *Fundulus heteroclitus* from below against white and against black backgrounds did not result in the same color changes evoked in illumination from above, therefore implying a fixed structural difference in the retina. This difference apparently lies in the structure and distribution of the rods and cones in the dorsal and ventral portions of the retina (Butcher, 1937).

tested experimentally by subjecting animals to illumination from below against the gray background (see "Methods").

The results from this series are not entirely conclusive. Of 7 eyes studied 3 showed uniform migration of the pigment, the position of the

EXPLANATION OF PLATE I

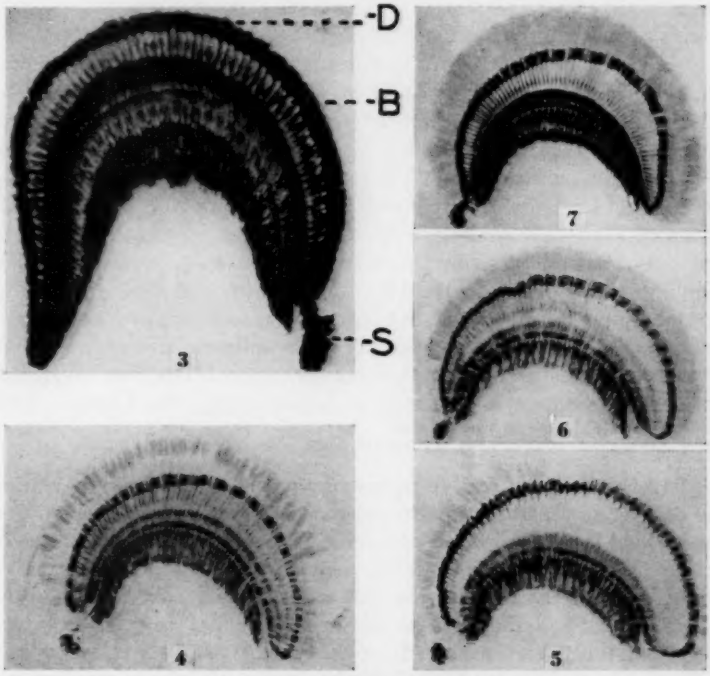

FIG. 3. An enlarged photograph of a completely light-adapted retina, showing the landmarks used in measuring the position of the distal pigment. *D*, distal pigment cells; *B*, basement membrane; *S*, dorsal pigment spot.

FIGS. 4 AND 5. Retinas from Series *A*-1 and *B*-1 respectively. The intensity of incident light was the same in both cases, but the backgrounds were different. Not only is the position of the distal pigment unequal in the two eyes, but that in the dorsal region of the retina has migrated to a greater extent toward the basement membrane.

FIGS. 6 AND 7. Retinas from Series *B*-2 and *A*-2 respectively.

distal cells being the same in the dorsal and ventral halves of the retina; in three others the distal retinal pigment had migrated to a greater extent in the dorsal portion of the eye; and one eye was irregular. Inasmuch as the pigment had migrated to a greater extent in the dorsal

portion of the retina in the eyes of all animals illuminated from above (except in series *A*-3 where the eyes were completely light-adapted) the fact that in some of the animals illuminated from below migration of the distal retinal pigment was uniform may indicate a slight direct response of those cells to light. This cannot be stated with any certainty, however, and requires further study.

Discussion

It is generally stated that, within certain limits, chromatophoral activity in animals on particular backgrounds is largely independent of the intensity of incident light. Recent studies have been indicating a certain relation between the visual field and the chromatophore response. Thus, Sumner (1933) found that covering the lower halves of the eyes in *Fundulus parvipinnis* resulted in darkening of the animals even on white backgrounds, while darkening the upper half of the field of vision gave only a slight dispersion of melanophore pigment in fishes on white backgrounds. Hanström (1937) obtained similar results in opaquing the dorsal and ventral portions of the retinas of *Palaemonetes vulgaris*. Brown (1936) found that in the minnow, *Ericymba buccata*, "the average diameter of the melanophore masses (therefore the degree of darkening) appears to vary in a directly proportional fashion with the ratio, $\frac{\text{incident light}}{\text{reflected light}}$, which reaches the eyes of the fish."

All these results resolve themselves into a differential stimulation of dorsal and ventral retinal receptors. In *Leander* a similar condition must hold for the migration of the retinal pigment. The migration of the retinal pigment, however, is assumed to regulate the amount of light which arrives at any particular rhabdome. (In dim light the distal position of the pigment allows oblique rays to pass; in bright light oblique rays are excluded, only those rays parallel to the long axis of the ommatidium reaching the rhabdome.) The above experiments, therefore, do not determine whether the different extents to which the retinal pigment migrates in the dorsal and ventral halves of the eye contribute to the differential stimulation of dorsal and ventral photoreceptors or whether they are the result of such a differential stimulation.

Summary

1. On a constant background the amount of migration of the distal retinal pigment in *Leander adspersus* is directly proportional to the intensity of incident light, within the limits of light- and of dark-adaptation.

2. Under the conditions of illumination used in these experiments the position of the distal retinal pigment is in direct proportion to the ratio, $\frac{\text{intensity of incident light}}{\text{intensity of reflected light}}$.

3. In all eyes, except those completely light- and totally dark-adapted, the pigment is found to migrate to a greater extent in the dorsal half of the retina. The reason for this variation in position of the pigment of the dorsal and ventral portions of the retina is not clear.

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INFLUENCE OF ASSOCIATION ON THE SEXUAL PHASES OF GASTROPODS HAVING PROTANDRIC CONSECUTIVE SEXUALITY

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The studies of Orton (1909, 1912, 1922), Kleinsteuber (1913), Giese (1915), Gould (1917*a*, 1917*b*, 1919), Coe (1935, 1936, 1938*a*, 1938*b*), Ishiki (1936) and others have shown the occurrence of protandric consecutive sexuality in all members of the pectinibranch family Calyptraeidae (or Capulidae) which have been investigated hitherto, making it appear probable that this type of sexuality is characteristic of the entire family.

The conditions which inaugurate the change from male to female phase, however, have not yet been fully elucidated. Moreover, Gould (1919) concluded that the development of the functional male phase in *Crepidula plana* depended upon the secretion of a masculinizing substance by an older individual with which the sexually immature or "neuter" individual was associated. Actual contact was deemed unnecessary, since it was assumed that the secretion could pass through the water. This conclusion has been widely quoted in the textbooks, although it has been shown recently (Coe, 1935, 1936) that solitary or isolated young of that species may under favorable conditions assume the functional male phase when living entirely isolated from other individuals. Nevertheless, the masculine characteristics when already established may be accentuated and prolonged in that and other species by the association of the male with a female.

Ishiki (1936) concluded that in both *Crepidula walshi* and *C. aculeata* some stimulation of a chemical nature "is carried toward the small individual from the female" which prolongs the male phase as long as association with the female continues. The present writer (1938*a*), on the other hand, discredits the idea of a masculinizing hormone passing from the female to the associated male and assumes that the accentuation and prolongation of his masculine characteristics result from hormones secreted within his own body as the result of stimuli received by his nervous system from the body of the female through the sense

[1] Contribution from the Scripps Institution of Oceanography, New Series, No. 29.

organs in his penis and tentacles. Such sensory stimuli doubtless dominate his autonomic responses during copulation as well.

It has been shown (Coe, 1936) that the three common species of *Crepidula* (*C. fornicata*, *C. convexa* and *C. plana*) on the Atlantic coast respond in a somewhat similar manner to the influences of association, although the individuals of the three species differ so greatly in their habits. It has seemed desirable, therefore, to extend these studies to other species in order to determine more fully, if possible, the essential character of the responses observed in nature and experimentally.

There are some ten or eleven valid species of the genus *Crepidula* reported from the Pacific coast. Four of these (*C. onyx*, *C. lingulata*, *C. adunca* and *C. nummaria*) form the basis for the investigation here

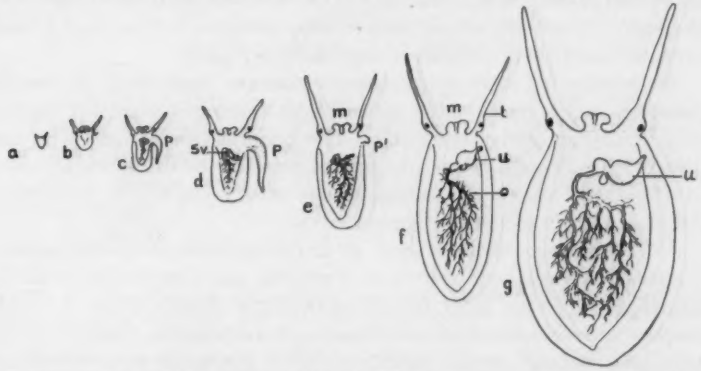

FIG. 1. Successive sexual phases in *Crepidula nummaria*, showing the development and absorption of the penis and the reorganization of the reproductive system preceding the female phase; *a*, recently hatched young; *b*, early male phase, with primordium of penis behind right tentacle; *c*, young functional male; *d*, mated male; *e*, transition stage; *f*, female phase, dwarf form; *g*, female when growing on unrestricted surface; *m*, mouth; *o*, ovary; *p*, penis; *sv*, seminal vesicle; *u*, uterus and seminal receptacle; *t*, tentacle.

reported. Each of these species can be obtained in abundance for experimental studies at all seasons of the year. All of them agree in having protandric consecutive sexuality, but each species differs in the associative behavior of the two sexual phases and in their responses to experimental conditions in the aquarium.

In these species, as in others previously studied (Gould, 1917*a*, 1917*b*; Coe, 1935, 1936, 1938*a*; Ishiki, 1936) the male phase becomes functional at an early age, when the body has reached only a small fraction of its definitive size (Fig. 1). After spermatogenesis has commenced a long, muscular penis grows out from the neck behind the right tentacle (Fig. 1). By means of this organ the sperm which have

been stored in the seminal vesicles are transferred as opportunities permit to the uterus and seminal receptacle of an older individual in the female phase (Fig. 1).

During the transition from the male to the female phase the residual spermatic cells are cytolyzed and such small ovocytes as may have become differentiated, but not cytolyzed, during the process of spermatogenesis increase rapidly in size and soon begin the deposition of yolk. The gonad thereby changes from a spermary to an ovary. Sometimes this change takes place rapidly but often there is a prolonged period of apparent inactivity, during which the gonadal follicles contain only relatively few ovogonia. In the latter case the body may grow rapidly while the genital duct is being transformed into an oviduct by the absorption of the seminal vesicles and the terminal portion enlarged and extended to form the uterus and seminal receptacle (Fig. 1). Under favorable conditions ovogenesis then follows rapidly.

Accompanying the morphological changes from male to female phase are fundamental alterations of an appropriate nature in the individual's instinctive behavior. The activities characteristic of the male are supplanted by such as are concerned with the formation and attachment of the egg capsules and the provisions for the protection and oxygenation of the developing larvae.

Unfavorable conditions, such as forced movements, accumulation of waste materials, lack of food or of oxygen, abnormal temperature or parasitism, when not fatal, terminate the male phase without the compensatory development of the female characteristics, leading to an essentially neutral sexual condition. If a favorable environment is restored the transformation to the female phase is resumed, but not otherwise. Occasionally the male phase may be aborted and the female functional. Excessive infestation by trematode larvae may also result in the almost complete destruction of the gonad after the female phase has become established.

Since only fragmentary accounts of the biology of these species have been published it seems advisable to discuss each of the four species separately, particularly in regard to the sexual phases and reproductive behavior.

Crepidula onyx Sowerby

(*C. rugosa* Nuttall)

This species closely resembles *C. fornicata* of the Atlantic coast—but now introduced and established on the Pacific coast—both in appearance and in habits. Pyramidal groups of three to sixteen or more individuals are often found in muddy bays. In such groups the basal individual is attached to an empty shell of the same or of some other

species of mollusk. This and one or more of the superimposed individuals are usually in the female phase, while transition forms, sedentary males, supplementary males and sexually immature individuals may be superimposed in the sequence named (Fig. 2, *a*).

The supplementary males move about from one female to another, while the sedentary males retain the same position permanently with respect to the immediately underlying individuals. Nevertheless, these sedentary males are capable of sufficient movement to enable them to fertilize females not immediately adjacent. They return to their original positions after copulation has been accomplished or at the approach of daylight, for the crepidulas are largely nocturnal in their activities.

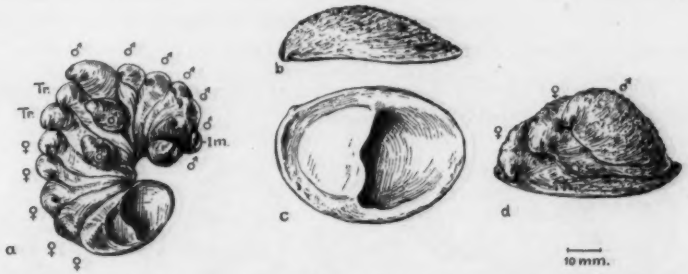

FIG. 2. *Crepidula onyx; a*, typical form, large group consisting of seventeen individuals from intertidal mud flat; the group includes five basal females, two transition individuals and seven superimposed functional males, with two small supplementary males and one sexually immature young (*Im*); *b*, large, isolated female of the roughened, flattened form (*rugosa*); *c*, same from ventral surface of empty shell; *d*, rugosa form, small group consisting of two basal females and one superimposed male. Scale indicates natural size.

Before beginning transition to the female phase the sedentary males often reach a length almost equal to that of some of the underlying females. In such males the transition period is very brief (Fig. 6).

The supplementary and solitary males, on the other hand, as is also the case with many mated males that are isolated experimentally when of small size, have a much longer transition period. During this period they grow considerably but not sufficiently rapidly to equal the sedentary males in size (Fig. 6). Growth also continues after the female phase is reached.

Another habitat form has a flat, oval shell and has been given the varietal name *rugosa* because of the roughened surface (Fig. 2, *b*, *c*, *d*). Individuals of this form occur singly or in groups of two, three or four, usually attached to *Pecten* or other large shells, or to rocks or spiles. These often become much larger than those living on muddy bottoms.

Females may exceed 60 mm. in length, while in mated or associated individuals the male phase may continue until the shell has a length of 35 mm. or more. A large proportion of the males, however, are solitary and transform to the female phase when relatively small. In other habitats only dwarf individuals are found.

In this species the accentuation and prolongation of the male phase in associated or mated individuals as compared with those which remain supplementary or solitary either by chance or by choice is very great (Fig. 6). Individuals of all ages are remarkably hardy, living for many months in the aquarium if sufficient food is available. Isolation of mated males usually, but not in all cases, terminates the male phase and inaugurates the female phase promptly.

Crepidula lingulata Gould

(*C. dorsata* Broderick)

This species likewise occurs in several different forms as the result of various environmental conditions.

Fig. 3. *Crepidula lingulata*. *A*, mated pair. *B*, female with two not closely associated males. *C*, female with mated male removed, showing "scar" indicating that the male may sometimes remain attached for a considerable period. *D*, shell of female from ventral side to show character of internal partition.

The male phase commonly becomes functional when the young individual has reached a length of 3 to 5 mm., while the transition stages occur at about 8 to 11 mm. The average individual assumes the female phase when about 9 to 12 mm. long, after which growth usually continues until a length of 18 to 20 mm. is reached. An occasional female may be more than 25 mm. long.

It would appear that only temporary matings are the rule in this species since not more than 5 per cent of the mature females observed were accompanied by mated males (Fig. 3) at the time of collection. Only about 5 per cent of the other females showed scars to indicate where the males had been formerly attached (Fig. 3, *c*). All of them, however, had sperm in their seminal receptacles, indicating that temporary matings, presumably nocturnal, had taken place. The males were scattered about in the neighborhood but only occasionally in the immediate vicinity of the females. Consequently in this species no

evidence is available as to the effects of association on the sexuality of the male. None of the functional males observed exceeded 12 mm. in length.

CREPIDULA NUMMARIA GOULD

(*C. nivea* C. B. Adams)

This species also is very variable in form as a response to the habitat of the individual. When growing within the narrow space inside an empty shell of *Tegula* or other small univalve, the adults in the female phase are very slender and only 14 to 16 mm. in length (Fig. 4, *a*, *b*). When attached to the surface of a large shell, stone or other solid object, however, the shell becomes broadly oval (Fig. 4, *c*, *d*) or even nearly circular, as was the case with Gould's type specimen, and may sometimes exceed 25 mm. in length.

There can be no further doubt as to the specific identity of the two forms, since the newly hatched young of the slender form have grown into the broad form *nivea* when reared in the aquarium at the Scripps Institution of Oceanography.

FIG. 4. *Crepidula nummaria*. *a*, *b*, mature females, dwarf form, with mated males, living in small univalve shells; *c*, *d*, same when living on surface which allows unlimited space for growth. Scale shows natural size.

The young often reach the functional male phase within a few weeks after hatching, when the body is less than 3 mm. in length (Figs. 4, 6). Others remain sexually immature until they are 6 to 8 mm. long. The sexual transformation commonly takes place at a length of 7 to 10 mm.; most individuals more than 10 mm. long are in the female phase (Fig. 6). The successive stages in the development and transformation of the reproductive system in the two sexual phases are shown in Fig. 1. The early gonad is less distinctly ambisexual than in the other species investigated, since very few ovocytes become differentiated until toward the end of the transition period.

The males are not usually found in the mating position on the females (Fig. 3) but remain associated nearby or in contact until afforded opportunities for copulation. The influence of association in the accentuation and prolongation of the masculine characteristics becomes evident when series of associated and solitary males are com-

pared (Fig. 6). Unfavorable conditions postpone sexual maturity as well as terminate the male phase and delay the differentiation of the female phase.

Crepidula adunca Sowerby

This species resembles *C. convexa* of the Atlantic coast (but now established on the Pacific coast) in its habits (Coe, 1936).

Conklin (1897, 1898) studied the reproduction and development of this species and Moritz (1938) has described the anatomy of the young male. The mated males retain the mating position for a long period (Fig. 5) but usually leave the female at the beginning of the transition phase.

Fig. 5. *Crepidula adunca.* Outlines showing mated females, with associated males; (*a*) female with male of average size; (*b*) female with relatively large male; (*c*) female with mated male and supplementary male; (*d*) two superimposed females each with mated male.

The young may reach the functional male phase within a few weeks after hatching, sometimes when less than 4 mm. in length. The sexual transformation commonly begins when a length of 9 to 10 mm. is reached (Fig. 6). The influence of association or mating on the accentuation and prolongation of the male phase is less evident in this species than in most others that have been studied, although the tabulation of many observations shows a significant difference between solitary and mated individuals (Fig. 6).

Comparison with Other Species

Three of these species, *C. onyx*, *C. nummaria* and *C. adunca*, demonstrate the influence of association on the accentuation and prolongation of the male phase (Fig. 6). In the fourth species, *C. lingulata*, no such evidence has been obtained. In none of these species, however, nor in any member of the family Calyptraeidae thus far investigated, is such association a prerequisite for the functional development of the male phase, but only for its accentuation and prolongation.

The three species of *Crepidula* on the Atlantic coast, *C. convexa*, *C. plana* and *C. fornicata*, show the same influence of association as do

the three Pacific coast species mentioned (Coe, 1936, 1938*a*). This is likewise true of the two Japanese species studied by Ishiki (1936) as well as of *Crucibulum spinosum* (Coe, 1938*b*).

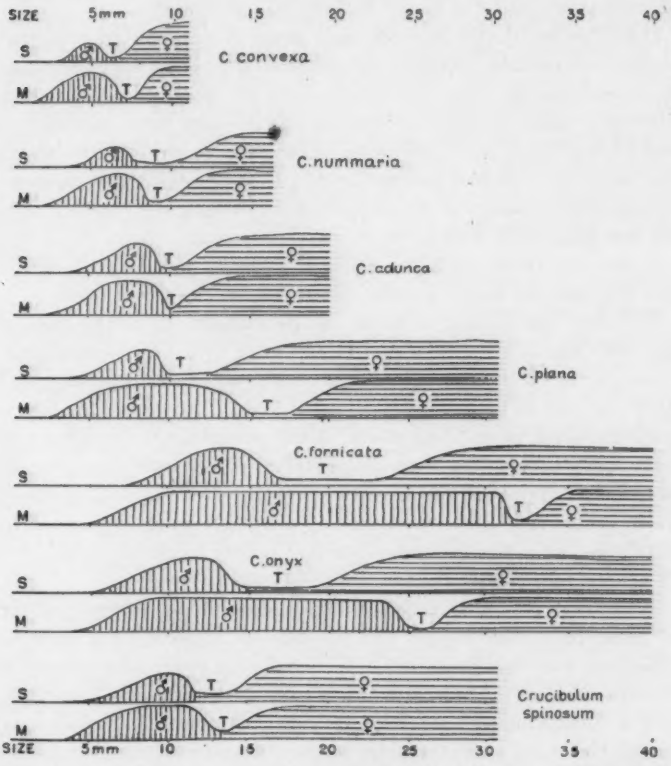

FIG. 6. Diagrams indicating the relative duration of each of the sexual phases in isolated or solitary males (*S*) as compared with associated or mated males (*M*) in six species of *Crepidula* and in *Crucibulum spinosum*. In each case the height of the curve indicates the relative development of the reproductive system. The sizes given indicate the lengths of the shells. For each species it will be noted that the solitary individuals have a briefer functional male phase and a longer transition period (*T*) than the corresponding associated individuals. All the diagrams are drawn at the same horizontal scale, although the heights of the curves are not strictly relative for the different species.

The comparative development of the gonads in the sexually functional and transition phases in solitary and mated or associated males in six species of *Crepidula* and in *Crucibulum spinosum* is shown diagram-

matically in Fig. 6. All are drawn at approximately the same scale as regards body length. The accentuation and prolongation of the functional male phase in associated or mated (*M*) as compared with isolated or solitary (*S*) individuals are characteristic for each species. The transition period is longer in solitary individuals.

It will be noted also that the male phase is inaugurated at a smaller size and a presumably younger age in mated than in solitary individuals. It has been assumed that the mated young develop functional maleness earlier as a result of their association but it seems more plausible that those young which reach the male phase earliest are the ones that are most likely to be mated earliest by virtue of the early development of their masculine instincts which lead them to seek the female. Those with less masculine characteristics are presumably more likely to remain solitary (Coe, 1938*a*). Indeed, it seems not improbable that there may also be minor genetic differences in the two groups.

Whether the mating is to be (a) permanent, as is the case with some individuals in *C. onyx* and *C. fornicata* or (b) long-continued, as may occur in *C. convexa*, *C. adunca* and *Crucibulum spinosum*, or (c) highly variable in length, as in *C. plana* and *C. nummaria*, or (d) comparatively brief, as in *C. lingulata*, is also presumably dependent upon genetic differences. Both specific differences and individual peculiarities in instinctive behavior are doubtless involved, as well as environmental influences. Also the question of chance as distinguished from choice must be taken into consideration before a conclusion is reached as to the actual significance of the behavior observed.

With these precautions in mind the nature of the influence whereby the presence of the female accentuates and prolongs the functional male phase may be considered. Experimental evidence indicates that the empty shell of the female does not have the same effect (Coe, 1938*a*). That a masculinizing hormone or other chemical substance passes through the water from the older to the younger individual or from the female to the associated male, as Gould (1917*b*, 1919) and Ishiki (1936) have supposed, seems improbable for the reasons stated in the introduction to this article.

There can be no doubt, however, that the associated male receives stimuli from the female through his sense organs in the penis and tentacles. These sensory stimuli not only affect the behavioristic instincts of the male but they also elicit responses at the time of copulation, resulting in the transfer of sperm to the seminal receptacle of the female. They doubtless also influence such other parts of the male's autonomic system as are concerned with the accentuation and pro-

longation of his masculine characteristics, presumably through the secretion of hormones within his body.

In the immature male the activation of the spermary and the inauguration of spermatogenesis precede the growth of the penis. Moreover, cessation of active spermatogenesis precedes the absorption of the penis at the termination of the male phase whether such termination be due to isolation, to unfavorable environment or to the internal changes which take place when a certain degree of maturity is reached. Sexual metamorphosis in these mollusks resembles other types of metamorphosis in animals in that the juvenile (male) phase can be abbreviated or prolonged experimentally but the sequence of the sexual phases cannot be reversed. It may be assumed that all these changes are mediated by internal secretions.

It is significant that in these species of *Crepidula* and *Crucibulum*, as well as in other described cases of protandric consecutive sexuality in planarians, nemerteans, oligochaetes, chaetopods, echinoderms, crustacea, pulmonate gastropods, oysters, clams, teredos and other forms the activation of the male constituents of the primary ambisexual gonad always precedes the development of such secondary morphological and behavioristic sexual characteristics as are peculiar to the functional male phase. The external environment in many cases determines the extent of this phase. The termination of the male phase and the metamorphosis to the functional female phase are similarly correlated with the activation of the ovarian constituents of the gonad. Such also occurs in the cases of alternative sexuality, as in the oviparous oysters, and of rhythmical sexuality, as in the larviparous oysters. That these developmental and transformational activities are mediated by the secretion of growth-stimulating substances to which certain localized groups of cells are attuned seems highly probable.

Summary

1. The functional sexual phases of four species of Pacific coast gastropods with protandric consecutive sexuality have been investigated with regard to the conditions which control the change of sexual phase and particularly with respect to the influence of association or mating on the individual's sexuality.

2. In none of these species nor in six species previously studied is association a prerequisite for the development of the functional male phase, although isolation of these males after a prolonged mating period usually terminates the male phase promptly.

3. In nine of the ten species studied intimate association or permanent mating of the young male with one or more individuals in the female phase tends to accentuate the masculine characteristics and to postpone sexual transformation by prolonging the male phase. In five of these ten species the influence of association is very conspicuous; in four it is apparent but not conspicuous; in only one is the evidence inconclusive.

4. There seems to be no evidence that the accentuation and prolongation of the male phase by association is due to any masculinizing chemical secretion or hormone which passes through the water from the body of the female to that of the male, as some investigators have assumed; it seems more probable that stimuli received by the male through the sense organs in his penis and tentacles during association with the female are of such a nature as to influence his masculine characteristics, presumably by the mediation of hormones secreted within his own body.

5. Sexual maturity and sexual metamorphosis in these mollusks, like other types of metamorphosis in animals, are subject to environmental influences and can be accelerated, postponed or inhibited experimentally, but the sequence of sexual phases cannot be reversed.

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PHYSIOLOGY OF REPRODUCTION OF OSTREA VIRGINICA

II. Stimulation of Spawning in the Female Oyster

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Introduction

The second part of the investigations on the physiology of reproduction of the oyster (see Galtsoff, 1938) deals with the conditions which provoke ovulation and the discharge of eggs by ripe females. The work was carried out at the U. S. Fisheries Laboratory at Woods Hole. Live material for the experiments was obtained from Onset and Wellfleet, Massachusetts; Long Island Sound and the Chesapeake Bay. No difference was observed in the reactions of oysters from these three localities.

Ecological literature provides numerous data suggesting a correlation between the breeding periods of various marine animals and sea temperature (Plymouth Marine Invertebrate Fauna, 1904; Lo Bianco, 1909; Zernov, 1913). An attempt to analyze a mass of these observations from a physiological point of view was made by Orton (1920), who grouped all marine organisms into three classes: (*a*) those which breed at a definite temperature characteristic for the species throughout its range of distribution; (*b*) those in which breeding is influenced by a change in temperature, either at the maximum or the minimum for the locality; and (*c*) those which breed the year round. Yonge (1930) has demonstrated that some of the animals of the latter group breed for the greater part of the year not continuously, but periodically. Numerous observations on *O. virginica* (Ryder, 1887; J. Nelson, 1904; Moore, 1907; Churchill, 1920; Gutsell, 1924; Prytherch, 1928; Galtsoff, Prytherch and McMillin, 1930) have shown that this species spawns at rising temperature and therefore should be included in the second group of Orton's classification. Temperature about 20° C. was considered by these observers to be the lowest at which spawning takes place. Nelson (1928*b*) states that "once this 'critical temperature,' 20° C., is reached a 'trigger' mechanism is released which requires some hours for its consummation, and that the speed of the underlying processes is governed by temperature." Ecological evidence upon which this conclusion is based consists in daily records of the occur-

rence of oyster larvae in plankton, temperature data and weekly examination of oyster gonads. Its inadequacy in dealing with the physiological problem is apparent for neither the act of spawning nor the exact temperature of water, which is supposed to induce it, were observed in the field. Because the daily fluctuations in temperature in shallow water may exceed several degrees, the concept of "critical" temperature in nature has very little meaning unless it is specified whether the daily average or maximum or minimum is considered. Further observations by Nelson (1931) made in England show that *O. virginica* can be induced to spawn at 19.1° C. This throws further doubt on the validity of the view that 20° C. is the "critical" temperature in spawning of this species.

That temperature is not the only factor which governs the propagation of the oyster is shown by the observations of Hopkins (1931) and Loosanoff (1932) in Texas and Virginia. The breeding season in these waters extends over a period of several months during which the temperature remains well above 20° C. Since outbursts of spawning occurring during this time do not coincide with the rise or fall of temperature, other stimuli must be responsible for provoking sexual activity. Inasmuch as field observations fail to throw light on their nature, an experimental investigation conducted under carefully controlled laboratory conditions appears to be more promising.

Stimulation by Temperature

The question whether temperature alone can stimulate ovulation and the discharge of eggs from the pallial cavity of an oyster was studied in the two sets of laboratory experiments designed to determine the effects (1) of a prolonged exposure at increased constant temperature, and (2) of a quick rise of temperature from about 20° C. to 34°–35° C.

In the first series of experiments ripe females, kept in a live car in the bay, were brought to the laboratory and placed in water warmed by means of a no-lag immersion heater, to a desired degree. By using a mercury thermoregulator the temperature was kept constant within ± 0.2°. To avoid the effect of chemical stimulation by sperm which might have been present in the laboratory water supply, all the water was filtered through a 2½ inch layer of asbestos. If the experiments lasted more than 12 hours, running sea water was provided, otherwise the water was aerated and stirred but not changed. The beginning and end of the spawning reaction were determined from the kymograph records of shell movements. The presence of eggs which were collected from the bottom of the tank and kept under observation for

24 hours served as a supplementary proof that spawning took place. In all the cases they were unfertilized and failed to develop, indicating the absence of sperm in the water. The time which elapsed from the moment the oyster was exposed to a given temperature until the beginning of spawning is designated as the latent period of the reaction. The temperature of water in which the oysters were kept before the experiments varied between 19.5° and 20.6° and the range of temperatures to which they were exposed during the experiments extended

TABLE I

Stimulation of spawning by temperature

Experiment number	Date	Temperature ° C.			Latent period	F
		Before	During	Increase		
					minutes	*hrs.* × ° *C.*
437	July 9, 1934	20.6	22.5	1.9	591	19.7
437a	July 9, 1934	20.6	22.5	1.9	659	20.87
399	July 15, 1932	20.0	22.5	2.5	3060	127.50
498	July 5, 1935	20.3	25.0	4.7	35	2.74
498a	July 5, 1935	20.3	25.0	4.7	12	0.94
317	July 7, 1931	19.9	25.3	5.4	250	22.50
433	July 7, 1934	21.4	25.4	4.0	501	33.40
433a	July 7, 1934	21.4	25.4	4.0	500	33.30
321	July 9, 1931	19.9	25.0	5.1	22	1.87
325	July 10, 1931	19.5	24.5	5.0	65	5.42
492	Sept. 5, 1934	20.4	26.0	5.6	71	6.63
432	July 6, 1934	21.0	27.5	6.5	338	36.62
432a	July 6, 1934	21.0	27.5	6.5	340	38.83
318	July 8, 1931	19.9	28.0	8.1	32	4.32
319	July 8, 1931	19.9	28.5	8.6	55	7.88
322	July 9, 1931	19.9	26.0	6.1	257	26.13
320	July 8, 1931	19.9	30.0	10.1	42	7.07
438	July 10, 1934	20.6	30.0	9.4	405	63.45
438a	July 10, 1934	20.6	30.0	9.4	450	70.50
340	July 17, 1931	20.4	30.0	9.6	20	3.20

from 22.6° to 30.0° C. For the sake of convenience the results presented in Table I are arranged in four classes beginning with a temperature increase not exceeding 2.5° and ending with a ten degree rise. Many experiments in which oysters failed to respond to temperature stimulation are not included in this table, which contains only positive results. An examination of column 6 discloses the lack of correlation between the temperature and the latent periods of spawning reactions. Each class of experiments comprise two distinct groups of slow and

quick reacting oysters, the latent periods of which at 25° and above, fall either within 12 to 71 or between 250 and 501 minutes. Three experiments carried at 22.5° C. also show two groups of oysters with latent periods of about 600 and 3,000 minutes respectively. By examining the data presented in column 6 of the table, one can notice that the values of latent periods in different groups almost overlap. For instance, the minimum latent period of the first group (22.5°) is 591 minutes as compared with 501 minutes, the maximum latent period of the next higher temperature group. Plotting the latent periods against the temperature results in an erratic distribution of data showing an absence of any definite trend. Similar results are obtained if the amount of heat, *F*, received by the oyster and expressed as a product of the latent period multiplied by the degrees of increase in temperature, is considered. Examination of the last column of Table I shows wide fluctuation in this value and the absence of any definite correlation between it and the latent period.

Since under the conditions of the experiments, negative results have no meaning unless it is proved that experimental oysters were capable of spawning, every female that failed to respond to the increase of temperature was at the end of the experiment stimulated by sperm. Positive reaction was a proof that the female was physiologically ripe and that the lack of the response was due to the ineffectiveness of the stimulus. It was found that thermic stimulation fails in a larger number of ripe females. For instance, of the two females used in experiment No. 399, only one (shown in Table I) spawned at 22.5° C. Thirty-two hours of exposure at this temperature failed to stimulate spawning in the other one, which, however, copiously discharged eggs within a few minutes after the addition of sperm. In another experiment (not listed in Table I) three ripe females were taken from the tank in which the temperature during the previous week fluctuated between 18.5° and 19.5° C., and were placed in filtered sea water. The temperature was maintained at 22.6° but occasionally rose for a short time to 23.4°. Continuous kymograph records of shell movement of each of the females were obtained. The first oyster was kept under observation for 5 hours, 22 minutes; the second for 29 hours, 14 minutes; and the last one for 73 hours, 13 minutes. None of the oysters spawned during the experiment but all three shed eggs after sperm was added to the water (22.6°), the latent period being 16, 24, and 15 minutes respectively (Galtsoff, 1932). During the course of the investigations a large number of similar cases were recorded. Undoubtedly some of the oysters which were kept only for 24 hours at a constant temperature might have spawned if the experiments had been

continued for a longer period of time. This was, however, impractical and the majority of the experiments had to be completed within one day. The percentage of ripe females which under those conditions were not responsive to temperature stimulation remains therefore undetermined. Yet the fact that within a range from 22.5° to 30° spawning in some of the females can be stimulated by exposing them to a constant temperature, higher than that of their original environment, is well established.

In a second set of experiments carried out in the summer of 1937, the effect of rapid rise in temperature from about 20° C. to 34°–35° C. was studied. After the oyster was placed in the experimental tank the water was quickly heated by an electric Hotpoint immersion heater, which was immediately removed as soon as the thermometer had registered the desired temperature. Then the water was allowed to cool. Usually it required about 20 minutes to bring the temperature from 20° to 34° C. In several instances oysters spawned before 34°

TABLE II

Latent periods (in minutes) of spawning reactions stimulated by rapid rise of temperature

Temperature		Latent period			Number of observations
Before spawning	During spawning	Maximum	Minimum	Average	
18–21	32.0–32.9	40	22	33	3
18–20.5	33.0–33.9	20	20	20	2
18–22.5	34.0–34.9	48	17	25.5	12

had been reached. Out of 43 females used in these experiments, 17 or 39.5 per cent were stimulated to spawn; 24 or 55.8 per cent failed to react to temperature but were induced to spawn by the addition of sperm; and 2 or 4.7 per cent failed to respond to the combined action of both stimuli. The latent periods of the reactions (Table II) i.e., time elapsed between the moment the heat was turned on and the beginning of spawning, varied from 17 to 48 minutes. One can notice that the lowest average latent period occurs at the temperature between 33° and 33.9° C. On account of a small number of observations it is, however, impossible to state whether the observed differences have physiological significance and the temperature between the 33° and 34° is the optimum for stimulation of spawning in the female. At 35° and above, the adductor muscle contracts and the shell remains closed. With the drop of temperature to 34° the adductor relaxes again. No spawning was observed above 35° and oysters exposed for two minutes to temperatures between 35° and 37° and left after that

for thirty minutes at 32° to 33° failed to spawn but immediately discharged eggs upon the addition of sperm.

Sudden rise of temperature from 20° to 32°–35° appears to be a more effective stimulant than long-continued exposure to lower constant temperatures. The question arises whether the high temperatures used in these experiments occur in nature and are therefore significant in the propagation of the species. It is true that in northern waters the bottom temperature of the estuaries rarely reaches 27°–28° C. On the other hand, temperatures above 30° often occur in the coastal waters of the southern states. Hopkins has shown (1931) that in June–August the temperature over the oyster beds in Offats Bayou (Texas) remained above 30° and often exceeded 33° C. And further observations in other parts of the Texas coast, made by the Bureau of Fisheries in 1935, confirmed his findings. Furthermore, oysters exposed at low tide to direct sun rays are often heated to a much higher degree than that used in the experiments. For instance, temperatures from 29.2° to 35.8° were recorded by the author in the tissues of oysters exposed during a hot summer day above low water mark on tidal flats of Milford Harbor (Connecticut) while the water temperature in small tidal pools varied from 26.5° to 37.2°. Thus the temperature to which oysters were subjected in the laboratory lies within the thermal range of their normal habitat and the conditions of the experiment cannot be considered abnormal or even unusual.

Stimulation by Sperm

The sperm suspension used in all the experiments was prepared by mincing and squeezing 1 gram of ripe gonad tissue in a small amount of filtered sea water and diluting it up to 100 ml. Amounts varying from 0.03 to 5 ml. of this standard suspension were added to 30 liters of water in which the female oyster was kept. Mechanical stimulation was avoided by emptying the pipette in the vicinity of the stirrer, several inches from the body of the oyster. The water was agitated, aerated, and kept at constant temperature. This technique was followed throughout the investigation with the exception of a few special experiments in which sperm suspension was injected directly into the pallial cavity of the oyster.

It was shown in a previous paper (Galtsoff, 1930) that in a physiologically ripe female live sperm initiates a series of reactions which culminate in ovulation and rhythmical contractions of the adductor muscle. The first noticeable reaction of the oyster consists in one or several sharp contractions of the adductor usually within a few seconds after the addition of sperm (Fig. 1). This can be regarded as an ejec-

tion reaction caused by a chemical stimulation of the neural receptors of the tentacles and mantle. The presence or absence of this reaction is not a sign of the readiness of the female to spawn for the spawned-out and unripe oysters react in a similar manner and furthermore identical response can be obtained with any chemical substance to which the neural receptors of the oyster are sensitive. It is certainly not a part of the complex spawning reaction of the female.

The approach of spawning is manifested by the relaxation of the adductor muscle and decrease in the frequency of muscular contractions. The latent period during which these changes occur can be accurately measured from the kymograph records for the beginning of the spawning reaction is unmistakably marked by a series of rapid rhythmical contractions and the maintenance of a definite tonus level (Fig. 1).

The latent period determined in 101 experiments varies from 6 to 38 minutes. The frequency distribution of latency (Fig. 2) plotted for all

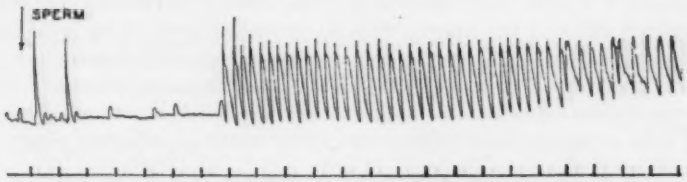


FIG. 1. Stimulation of spawning reaction in female *Ostrea virginica* by sperm. Time interval, 1 minute. Temperature 22.6° C.

these experiments, carried in different years and at temperatures ranging from 22.6° to 30.0° C., indicates the predominance of periods lasting from 9 to 12 minutes. In 78 per cent of the observations the latent period is less than 15 minutes. Its average value computed from all the records is 16.2 minutes. The latent period is independent of the concentration of sperm used in stimulating the reaction. This fact becomes apparent by examining Fig. 3 in which the latency indicated by ▲, is plotted against concentration. The present results confirm previous observations of Galtsoff (1930) in which no significant correlation was found between the two variables (Coef. of cor. 0.093 $\pm$ 0.106).

There is possibly a slight indirect correlation between the length of the latent period and temperature. Between 25.1° and 30° the peak of the frequency distribution (Fig. 2, blocked-in area) is between 6 and 9 minutes and average value of latent period (22 observations) is only 14.3 minutes as compared with the average of 16.7 minutes and the peak between 9 and 15 minutes at a temperature from 20.1° and 25°

(cross-hatched areas, 79 observations). Both groups, however, cover the entire range of variations.

The next problem deals with the effect of the concentration of sperm on intensity of spawning. If the stimulating effect is due to the direct action of spermatozoa on the receptors of the female one would expect that both the latent period and the intensity of spawning should vary

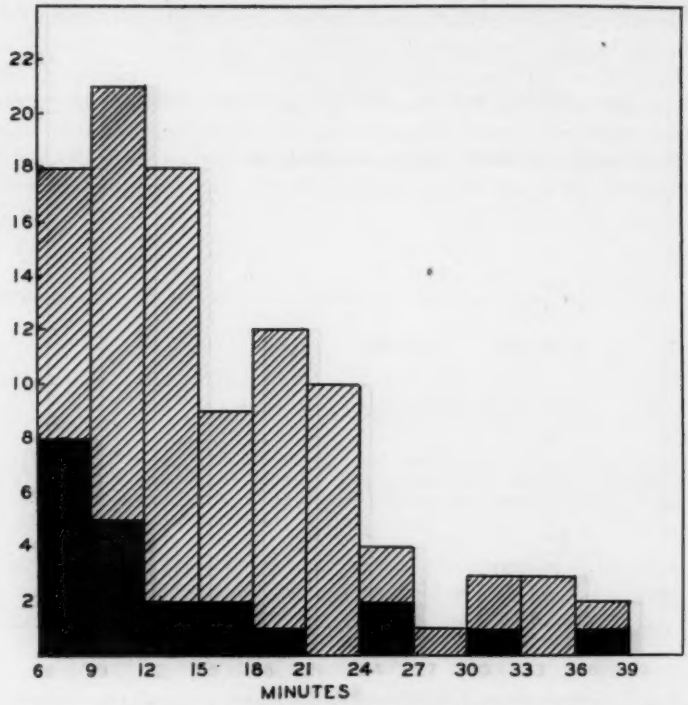


FIG. 2. Frequency distribution of the latent periods of spawning reaction in *Ostrea virginica*. Observations at temperatures between 25.1° and 30° C. are indicated by solid black areas; at 22.5–25.0°, by cross-hatched areas.

in proportion to the intensity of the stimulus, i.e., concentration of sperm. The duration of spawning reaction can be taken in this case as a measure of the intensity of the response. There is, however, no correlation between the two sets of data, as can be seen from Fig. 3, in which the duration of the reaction is indicated by □. Both the duration of the spawning reaction and its latent period are independent of the intensity of the stimulus.

The determination of the threshold of sensitivity of the ripe female to the presence of sperm was made by adding gradually increasing amounts of standard sperm suspension to the known volume of water in which the females were kept. The minimum dose of sperm capable of inducing spawning in the female (Fig. 4) was found to be between 0.02 and 0.03 ml. added to 30 liters of water. Counts made by means of a haemocytometer showed that the suspensions used in two experiments contained from 100 to 156 millions of live spermatozoa per 1 ml.

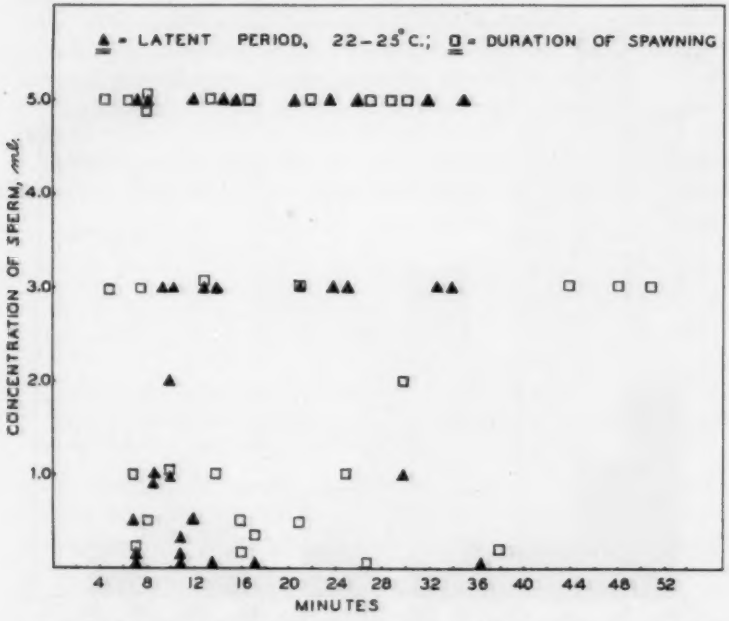


FIG. 3. Independence of latent periods and duration of spawning reaction in *Ostrea virginica* of the concentration of sperm.

or from 100 to 150 (in round figures) per milliliter of water surrounding the oyster. Since in other experiments the sperm suspension was standardized by weight, the exact figures of the number of spermatozoa per ml. probably have no definite significance, especially in view of the fact that the number of other cells present in suspension may vary depending on the state of the gonad development. Furthermore, oysters with greater pumping capacity may filter larger amounts of water and will therefore concentrate in their gills a greater number of

spermatozoa than can be accumulated in the same time by a smaller or less efficient mollusk. Present observations indicate, however, that no stimulating effect is to be expected if the number of spermatozoa in the water is below a certain minimum value. An inference can be made that to be effective the sperm must be present in sufficiently high concentration and that the proximity of oysters of both sexes on natural oyster bottoms is essential for the successful propagation of the species.

Immediately after spawning the female which may still contain a large number of ripe eggs becomes non-susceptible to further stimulation by sperm. This refractory period determined in a number of experiments was found to vary in different oysters from two to five days. Histological studies of the tissues of oysters preserved during this non-responsive period fail to reveal any conditions to which this state can be attributed. The ovaries contained a large number of ripe

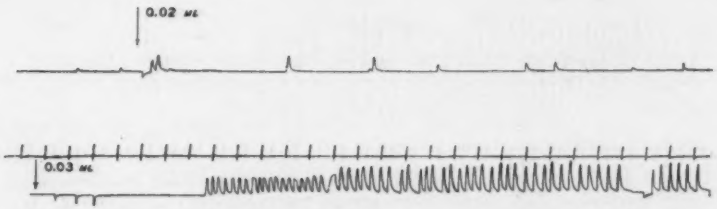


FIG. 4. Threshold concentration of sperm suspension in stimulating spawning reaction in *Ostrea virginica*. Vertical arrows indicate time when 0.02 ml. and 0.03 ml. of suspension were given. Time elapsed between the two tests, 1 hour. Middle line indicates one-minute intervals.

eggs lying free in the follicles and the oviducts appeared to be normal. In a short time the susceptibility is restored and the oyster can again be stimulated to spawn.

TEMPERATURE REQUIREMENT FOR STIMULATION OF SPAWNING BY SPERM

In the experiments designed to determine the threshold temperature required for stimulation of spawning, ripe females were treated with sperm at 18° C. After the lapse of two hours, during which continuous kymograph records of shell movements were taken, the water was changed, warmed to 20.5° and the oysters given a second dose of the same sperm suspension. In several instances typical spawning reaction took place, indicating that the threshold temperature is between 18.6° and 20.5°. It is of interest to note that at 18° C. some of the females reacted to the presence of sperm by increased muscular contractions. The up and down excursions of the muscle were, how-

ever, very small and the reaction was not accompanied by the discharge of eggs. The rhythmicity of these small contractions, not observed under any other conditions, suggested an abortive spawning reaction which failed to reach its full development. Upon raising the temperature to 20.5° C. and adding a second portion of the same sperm suspension, oysters spawned in a typical manner. Similar conditions but differing only in the temperature levels, were observed in *O. gigas* which in one of the experiments at 22.5° responded to the addition of sperm by small rhythmical contractions (Fig. 5, upper line) and spawned one hour later immediately after the temperature was raised to 25° C. (lower line).

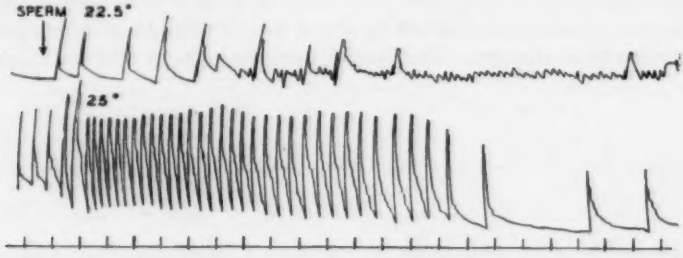


FIG. 5. Effect of temperature on the stimulation of spawning in *Ostrea gigas*. Abortive reaction at 22.5° (upper line); normal reaction at 25° C. Time interval, 1 minute.

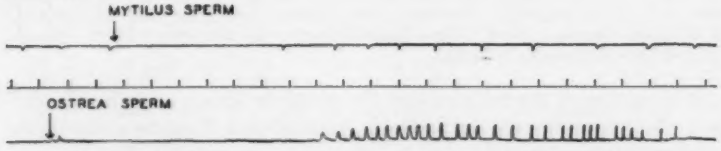


FIG. 6. Specificity of spawning reaction of *Ostrea virginica*. Time interval, 1 minute (middle line).

Can the temperature between 18.6° and 20.5° be regarded as critical for the spawning of *O. virginica*? In papers dealing with oyster spawning, the term "critical" is used in a very unqualified manner, without defining its exact meaning. Experimental evidence points to the fact that temperature around 20° causes no trigger-like effect on the spawning mechanism of the female. A large number of females capable of spawning were found to be unaffected by it and could not even be stimulated at this temperature by sperm. They required warmer water for the initiation of the spawning reaction. Thus we may conclude that the lower limit at which spawning may take place, i.e., between 18.6° and 20.5°, is not a critical temperature in

the sense that it initiates ovulation and the discharge of eggs in all physiologically ripe females. Its significance is in the fact that, under laboratory conditions, below this threshold temperature stimulation by sperm is not effective. This conclusion is corroborated by the following observations showing considerable variation in the lower temperature at which spawning can be induced. In the experiments made on June 17, 1936, at 27.5°, sperm failed to provoke the spawning reaction in ripe females which, however, spawned 5 hours later at 33.5° C. Both times the same sperm suspension was used. Spawning was brief with a very light discharge of eggs. In another experiment (Aug. 28, 1934) sperm had no effect on ripe females at 22° C. but stimulation was effected one hour later at 25.6°. An inference from these observations is that sexual responses to chemical stimulation depend on the state of the female organism which may require higher or lower temperature for effectuation of the spawning reaction.[1]

TABLE III

Effect of temperature on the stimulation of spawning by sperm in Ostrea gigas

Date	Temperature	Spawning	Temperature	Spawning
July 17	27.7	−	30.0	+
July 22	22.6	−	30.0	+
July 22	22.6	−	30.0	+
Aug. 8	28.0	−	30.0	+
Aug. 12	28.0	−	30.0	+
July 7*	26.0	−	28.0	+

* Two oysters.

Similar conditions were observed also in *O. gigas*. In the experiments summarized in Table III, each female was given 1 ml. of sperm suspension and kept for one hour at the temperature indicated in column 2. Then the water was warmed to the degree shown in column 4 and a second dose of sperm was administered. Success or failure to spawn is indicated by plus or minus sign. The results clearly show that ripe females non-responsive to sperm at one temperature react very easily to the same suspension at higher temperatures. In more than 30 laboratory experiments carried out with *O. gigas* at Woods Hole no spawning could be induced by sperm at temperatures below 25° C. Yet under natural conditions Elsey (1936) succeeded in stimulating spawning in a large number of oysters of this species at temperatures

[1] It has been found by Loosanoff that in June, 1938, oysters spawned in Long Island Sound at the temperature of about 17° C. The oyster larvae, however, failed to reach metamorphosis and perished. Some of these oysters taken by the author to the laboratory could not be stimulated to spawn at 20° C. and therefore the conditions which caused their early spawning in the Sound remained undetermined.

between 19.4° and 20.2°. Hopkins in a personal communication to the author states that on September 9, 1931, a female *O. gigas* spawned in the laboratory tank in Olympia, Washington, at 16.8° C. The rôle of temperature in controlling spawning in *O. gigas* is not clear, but the observations just quoted indicate a rather wide range under which it may occur. There is a possibility that as the season advances ripe unspawned females become more responsive to stimulation and spawn at lower temperatures than at the beginning of the breeding period. This point requires, however, verification.

Specificity of Spawning Reaction

The question whether the spawning reaction of the female oyster can be stimulated by any other agents besides the sperm of the oyster and an increase in temperature was studied in a series of experiments in which sperm of various mollusks and several hormones were tested. The results were negative. Ripe females were entirely indifferent to the addition of live sperm of *Venus mercenaria*, *Mya arenaria*, *Mytilus edulis*, *Modiolus modiolus* and *Pecten irradians*, but immediately reacted to the addition of oyster sperm (Fig. 6). The injection of thyroidin, theelin, and other hormones which were found to stimulate spawning of the male [2] were entirely ineffective.

So far as chemical stimulation is concerned, the spawning reaction of the ripe female appears to be specific and as present experiments indicate can not be provoked by any other agent except the sperm of an oyster. This specificity is not restricted, however, to the sperm of the same species for it has been found (Galtsoff, 1931) that stimulation of spawning in the female *Ostrea virginica* can be induced by the sperm of *O. gigas*, and vice versa. In both cases typical reaction ensues. Previous experiments (Galtsoff and Smith, 1932) prove that eggs of both species can be cross-fertilized and that the hybrids develop normally. It would be of interest to determine whether spawning in *O. virginica* and *O. gigas* can be provoked by sperm of other species of oysters. Nelson (1931) reports that sperm of *O. angulata* stimulates *O. virginica*, while Galtsoff (1931), when working in Honolulu, was unable to demonstrate any effect of the sperm of *O. commercialis* [3] on *O. virginica* and vice versa. The opportunity to make similar tests with other species has not yet occurred.

The biological significance of the specificity of the spawning reaction is obvious. The oyster discharges its eggs only when the right sperm capable of fertilizing them is present in the water. A non-specific response would result in a waste of the discharged material and event-

[2] The experiments with males will be fully discussed in a separate paper.

[3] In the above-mentioned paper reference is made to *O. cucullata*. It has been found since that the specimens used in the experiments were *O. commercialis*.

ually lead to the cessation of propagation and the extinction of the species.

Active Principle of Sperm Suspension

Attempts to determine the properties of the active principle of sperm suspension were made by testing its solubility in water and in various fat solvents and by ascertaining the effects of temperature and dehydration on its stability.

The first step was to determine whether live sperm is necessary to provoke spawning reaction. The question was answered in the negative by the experiments in which sperm killed by grinding with sand in sea water or in distilled water was used. Sand was purified by repeated washings in water, boiling in concentrated HCl, and washing again in distilled water until the disappearance of the last trace of acid. In killing the sperm the following procedure was used: A piece of ripe testis was ground for 30 minutes with ten times its volume of sand. Then a known quantity of filtered sea water was added, and the mass thoroughly stirred and set aside. As soon as the sand settled the supernatant suspension was pipetted and examined under the microscope. It contained no live sperm. Given to the ripe female kept in water at 23° C. it induced typical spawning, the latent period and the duration of which were within the normal range of the reaction stimulated by live sperm. Eggs discharged by the female were unfertilized and failed to develop during the following twenty-four hours. Identical results were obtained with sperm killed by grinding with sand in distilled water. The tests were repeated several times and always gave identical results which prove that killed sperm does not lose its stimulating power although its fertilizing ability has been destroyed.

The next step consisted in determining whether the active principle is present in the liquid phase of the sperm suspension or is located in the suspended particles. Filtration through tough Watman filter paper Nos. 44 and 52 failed to remove the spermatozoa and the filtrates were as effective in inducing spawning as the unfiltered standard suspension. Complete separation of sperm from the liquid phase has been accomplished by ultrafiltration, using ultrafine Zigmondy collodion membrane capable of retaining Congo Red particles. Samples of clear filtrates obtained in this manner were examined under the microscope and after being found free of suspended material were injected into the pallial cavity of the ripe females. After the lapse of one hour during which no spawning occurred, the residue was collected from the membrane and given to the oyster. As can be seen from Table IV, the filtrate lacked stimulating power, whereas the residue which still contained active sperm, was effective. In all four experiments eggs were fertilized and developed in a normal way.

It is concluded from the results of these experiments that the active principle of sperm suspension is not present in solution but is probably located in the bodies of spermatozoa. Unfortunately, a small number of blood cells and various elements of the connective and epithelial tissues are also present in the suspension. It is, however, unlikely that they are the bearers of the active principle, for it has been determined in a series of experiments that neither the blood nor the tissue juices of the male exert a stimulating effect on the female.

The insolubility of the active principle in the distilled water was demonstrated by ultrafiltering an extract prepared by grinding a piece of testis with sand. In these experiments 1.5 grams of ripe male gonad was minced in 25 ml. of distilled water and ground for 30 minutes in a glass mortar with 5 grams of purified sand. The sand was allowed to settle and a fine suspension was pipetted off and filtered through an ultrafine collodion membrane. The separate tests were performed with two ripe females kept at 20.9° and 21.5° C. Each time a fresh

TABLE IV

Effect of ultrafiltration on the stimulating power of sperm suspension

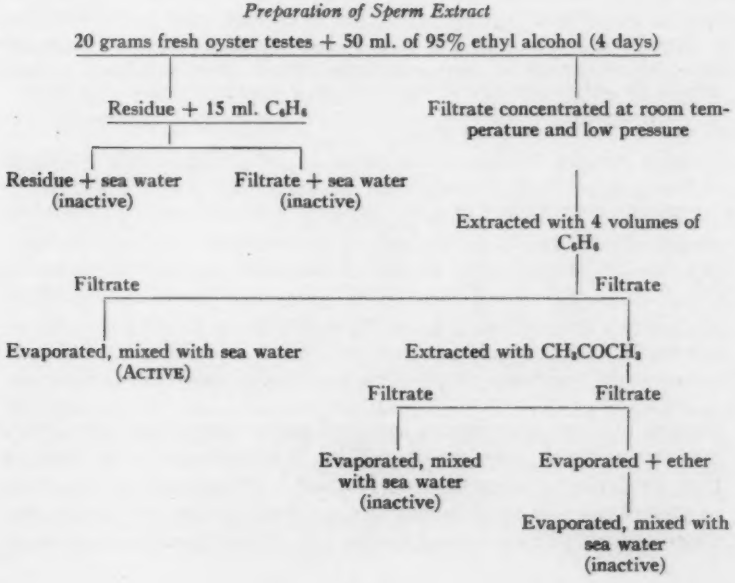
Date	Temp.	Membrane	Filtrate	Spawning	Residue	Spawning
	°C.		ml.		ml.	
June 19.....	23.8	Fine	2.5	−	1.0	+
June 29.....	26.8	Finest	2.5	−	1.0	+
July 21.....	25.0	Ultrafine	10.0	−	5.0	+
July 21.....	27.0	"	10.0	−	5.0	+

sample of gonad extract was prepared. The filtrates had no effect on the females which, however, reacted in a typical manner to the addition of the residue collected from the membrane. The discharged eggs were not fertilized and failed to develop. Identical results were obtained with sperm extracts in sea water. The experiments demonstrate that the active principle being insoluble in water remains within the cellular fragments of the gonad tissue from which it cannot be separated by this method.

EXTRACT OF ACTIVE PRINCIPLE

Since the active principle of sperm suspension was found to be insoluble in water, attempts have been made to extract it in alcohol, ether, benzene, chloroform, acetone, and other fat solvents. Out of many tests, successful results were obtained by employing the method similar to that developed by Koch (1931) and Gallagher and Koch (1929) for the preparation of the male hormone. From 15 to 20 grams of testes of several ripe oysters, after being freed as much as possible

from adjacent organs and connective tissue, were finely mashed in a glass mortar and extracted for four days at room temperature with about 50 ml. of 95 per cent ethyl alcohol. The alcoholic solution was filtered and the filtrate evaporated over night in vacuo until an aqueous suspension of lipins was obtained. Since some alcohol still was detectable by smell, about 15 ml. of pure benzene were added and the preparation evaporated almost to the consistency of paste. It was then extracted by repeated shaking with four volumes of benzene until the last benzene portion showed no color. The combined benzene portions were then evaporated to dryness under a vacuum and the extract, mixed with a small amount of filtered and boiled sea water, was injected into the pallial cavity of a ripe female capable of spawning. Out of five tests with this preparation four gave positive results. The reaction induced by the extract did not differ in any way from normal spawning reaction (Fig. 7). The latent periods varied between 10 and 20 minutes. The temperature of the water during the experiments fluctuated between 23.1 and 23.7° C. Attempts to purify the lipin mixture by dissolving it in acetone and separating the inert precipitate were not successful and all the fractions thus obtained had no specific action on females. Various steps of this process and results obtained with different fractions are shown in the following diagram.


Preparation of Sperm Extract
20 grams fresh oyster testes + 50 ml. of 95% ethyl alcohol (4 days)
Residue + 15 ml. C_6H_6
Filtrate concentrated at room temperature and low pressure
Residue + sea water (inactive)
Filtrate + sea water (inactive)
Extracted with 4 volumes of C_6H_6
Filtrate
Filtrate
Evaporated, mixed with sea water (Active)
Extracted with CH_3COCH_3
Filtrate
Filtrate
Evaporated, mixed with sea water (inactive)
Evaporated + ether
Evaporated, mixed with sea water (inactive)

In all the cases in which the preparation was found to be physiologically inactive, live sperm was given to the female and a positive spawning reaction was obtained, thus proving that the test oyster was capable of spawning. Small yields of active benzene extract precluded the possibility of further chemical analysis.

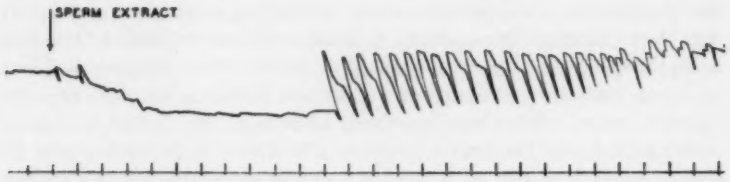


FIG. 7. Stimulation of spawning reaction in *Ostrea virginica* by the sperm extract. Time interval, 1 minute.

INSTABILITY OF THE ACTIVE PRINCIPLE

The active principle is thermolabile and loses its power after a brief exposure to temperatures ranging between 54° and 57° C. This fact was established in a series of experiments summarized in Table V.

TABLE V

Effect of heating on stimulating power of sperm suspension. Second column indicates the temperature at which sperm was tested. Third and fourth columns indicate the temperature used in heating sperm suspension and time of exposure.

Date	Temp.	Heated sperm suspension			Spawning	Normal sperm suspension	Spawning
				Dose			
	°C.	°C.	*minutes*	*ml.*		*ml.*	
July 8....	25.2	54	15	2	−	2	+
July 9....	23.0	55	15	1	−	1	+
July 10....	23.5	56	15	1	−	1	+
July 11....	23.4	58	15	1	−	1	+
July 13....	23.0	55	15	1	−	1	+
July 13....	23.0	55	15	1	−	1	+

In each of these experiments standard sperm suspension was divided into two portions. One was heated for 15 minutes, counting from the time the desired temperature was reached. The second portion served as control and was administered one, or in some cases, two hours after the treatment with the heated sperm. It is clear from the examination

of Table V that 15 minutes at a temperature of 54° and above suffices to destroy the potency of the active principle of sperm suspension.

Microscopic examination showed that all motion in the heated sperm had ceased and some of the spermatozoa were cytolized.

Sperm dried in the oven at 35° C. or at room temperature in vacuo completely lost its stimulating power and had no effect on ripe female oysters which, however, reacted to the addition of live sperm.

Probable Mode of Action of Sperm

Although the mode of action of sperm in stimulating spawning of the female is not known, certain speculation appears to be permissible. Our attention is first focused on the duration of the latent period which, as has been shown above, varies from 6 to 38 minutes. The length of time required for the realization of spawning is significant for it clearly shows the difference between this sexual reaction and an ordinary neuro-muscular response the latent period of which is measured in seconds. Furthermore, the duration of the latent period suggests that sperm may act through the digestive tract or after being absorbed by the gills and mantle of the oyster. The first possibility known to exist in *Platynereis megalops* (Just, 1914) has been investigated in the following manner. A large amount of fine carmine suspension was added to a number of actively feeding adult females. Every three minutes one of them was taken out, dissected and its stomach contents examined for the presence of carmine. It was found that at 22.6° C. a particle suspended in water reaches the digestive tract in about 7 to 12 minutes. It appears to be significant that the time required for the ingestion is about the same as that of the latent period of the spawning reaction. In another set of experiments attempts were made to prevent the sperm from reaching the digestive tract by plugging the mouth with beeswax and plastocele. Yonge (1928) used this method in a study of the absorption of glucose by *O. edulis*. In the present experiments Yonge's technique as well as several modifications of his original method was used without success. In all the cases, the females with plugged mouths spawned upon the addition of sperm. But the dissection of their stomachs revealed the presence of carmine particles which were added to the sperm suspension. This control measure proved the inefficiency of the method in blocking the entrance to the digestive tract. Since carmine particles were not prevented in reaching the mouth and stomach, it is reasonable to expect that sperm also reached the digestive tract. Thus, the experiments failed to throw light on the manner in which sperm acts on females. It is, however, obvious that in stimulating spawning the active principle of sperm does

not act directly through the chemical receptors of the tentacles and mantle which, as a rule, respond to chemical or mechanical stimulation within a few seconds.

On the assumption that rhythmical contractions of the adductor may be stimulated by some substance liberated during ovulation and distributed by the organs of circulation, blood of a spawning female was collected and injected in the tissues of another ripe female. In the first set of experiments blood was obtained from females stimulated to spawn by the addition of sperm. In the second series, the use of sperm was avoided and blood was obtained from the females spawning under the influence of temperature. Altogether eight experiments were performed with negative results. The females did not respond to the injection of blood of the spawning females but each of them responded in a typical manner to the addition of sperm. Negative results were also obtained with the blood and tissue juices of the male injected hypodermically or added to the pallial cavity of the female. In preparing the tissue juices the gonads were of course carefully avoided. In all the experiments oysters spawned immediately upon addition of sperm. The results show that of the various tissues of the male organism, testis alone is effective in provoking spawning.

It has been shown in the first part of this investigation (Galtsoff, 1938) that the spawning reaction, once initiated, proceeds in a typical manner irrespective of the nature of the stimulus. Evidence presented in this paper clearly shows that the latent period and duration of spawning reaction are independent of the intensity of the stimulus (degree of temperature or concentration of sperm) and that the reaction proceeds according to the "all-or-none" principle. The rôle of the stimulus apparently consists in setting in motion a chain of reactions which, once started, follow their course and cannot be stopped or inhibited.

Rhythmical contractions of the adductor muscle, typical for spawning reaction, are not caused by the direct action of sperm on the neuroreceptors of the female. This is proven by the fact that spawned-out and unripe females fail to respond to the presence of sperm. On the other hand, the activity of the adductor during spawning cannot be considered as a simple ejection reaction caused by the presence of eggs in the pallial cavity, for the contractions often precede ovulation and sometimes continue after the latter is stopped. The peculiar behavior of the adductor muscle must be attributed to the internal impulses originating in the female organism during spawning. It is a specific sexual reaction of the female. As the breeding season advances, the intensity of the spawning reaction gradually decreases. Completely

spent females are as a rule unable to respond to stimulation until the next breeding season. From a biological point of view, spawning reactions of the female oyster present an interesting example of specific physiological adaptation useful to a sedentary organism in providing better dispersal of eggs in the surrounding water and insuring greater chance for their fertilization. The advantages of the reaction in the survival of the species are obvious.

Summary

1. Spawning of the physiologically ripe female *O. virginica* can be induced by exposing it to a constant temperature a few degrees higher than that of its environment or by quickly raising the temperature of the water from about 20° C. to 34–35° C. The second method, which appears to be more effective than the first one, gave positive results in 39.5 per cent of the tests.

2. Sperm of the oyster is a more powerful stimulant of sexual reaction for it easily induces spawning in oysters which do not respond to temperature stimulation. In the laboratory experiments, 55 per cent of the oysters which failed to react to increased temperature immediately responded to the addition of sperm.

3. The latent period of spawning reaction varies from 6 to 38 minutes and is independent of the concentration of sperm. A minimum concentration of sperm of about 100–150 spermatozoa per ml. of sea water is, however, necessary to induce a reaction.

4. The lowest temperature at which spawning in *O. virginica* was induced lay between 18.6° and 20.5° C. This threshold temperature is not, however, a critical temperature in the sense that it induces spawning or makes the stimulation by sperm effective in all physiologically ripe females. The lowest temperature level of the spawning reaction varies in different oysters depending on their responsiveness to stimulation.

5. The spawning reaction of *O. virginica* is specific in the sense that sperm of other genera of mollusks cannot provoke it.

6. The active principle of sperm suspension is thermolabile and insoluble in water. It can be extracted in 95 per cent ethyl alcohol and benzene.

7. The spawning reaction follows the "all-or-none" principle and once started cannot be inhibited or stopped. Rhythmical contractions of the adductor are considered to be due to impulses originating in the female organism during spawning. They are not ejection reactions caused by the presence of eggs in the pallial cavity.

8. The possibility of the action of sperm through absorption by the digestive tract or through the gills is discussed.

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DISTOMUM LASIUM LEIDY, 1891 (SYN. CERCARIAEUM LINTONI MILLER AND NORTHUP, 1926), THE LARVAL STAGE OF ZOÖGONUS RUBELLUS (OLSSON, 1868) (SYN. Z. MIRUS LOOSS, 1901)

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Introduction

In one of his last publications, Joseph Leidy (1891) described a larval trematode, *Distomum lasium*, from the digestive gland and gonad of *Ilyanassa obsoleta* at Beach Haven, New Jersey. The account, although brief, was sufficiently complete and definitive to permit positive identification of the species. Leidy reported that *I. obsoleta* was very abundant on the mud flats near Beach Haven and that on dissecting a number he "found that one in five or six was infested with the larval distomas enclosed in sporocysts, often in large numbers, embedded in the liver and associated genital gland. The larvae always appeared in the distoma-form and never as a cercaria." He noted the shape and size of the sporocysts and of the tailless larvae, and for the latter he recorded the size of suckers, form of the excretory bladder, presence of spines on cuticula, and stylet in the dorsal wall of the oral sucker.

This species was reported by Linton (1915) from the same host at Woods Hole, Massachusetts. During the summers of 1909 and 1910 he had examined over one thousand specimens of *I. obsoleta* and a record of his dissections is presented. Apparently unaware of Leidy's earlier paper, Linton gave a description of the sporocyst and the cercariaeum, but did not name the species. A comparison of the descriptions of Leidy and Linton shows substantial agreement and leaves no doubt concerning the identity of the parasites collected in New Jersey and Massachusetts.

The account of Linton was confirmed and augmented by Miller and Northup (1926). These authors had likewise overlooked the report of Leidy and they named the larva *Cercariaeum lintoni*. In a study of the seasonal infestation of *Nassa obsoleta* with larval trematodes, they examined 8,875 individuals collected from a limited area in Quamquisset Harbor near Woods Hole, and found *Cercariaeum lintoni*

present throughout the year with the greatest infection in December and in June. It increased from 3 per cent in August to 7.5 per cent in December, decreased to 4 per cent at the end of February and began to increase in late May. The incidence of infection in June was not as great as that in December and it declined rapidly during the late summer. Miller and Northup reported on the number of snails from which mature larvae emerged and noted much variation between these figures and the total percentage of infection. They stated that the emergence of mature larvae "continues high from August to December and then apparently is lower until late July and August, at which time it approximates its former values." They discussed the possible effect of temperature and other factors on the seasonal infestation. Their description of the larva was brief and in certain particulars, e.g., the form of the alimentary tract and number of penetration glands, their observations were not correct.

The excretory system of *C. lintoni* was described by Africa (1930) from specimens collected at Cold Spring Harbor, Long Island, New York. He stated, "On account of the numerous refractive granules which obscured the capillary tubes and flame cells especially in the region behind the ventral sucker, great difficulty was experienced in making out these structures. In fact, the exact connection of the two flame cells of the posterior group has not been made out with certainty, but judging from the connections of the anterior apparently homologous groups, they are most likely drained by a common accessory tube just as their anterior neighbors are. It seems quite certain, however, that there are eight flame cells on each side, for in the study of more than a hundred individual specimens there has been no instance when a count of more than eight was made." Africa gave a diagrammatic figure of the excretory system and discussed the mechanism by which the pulsatile contraction of the vesicle is produced. In his figure he copied the incorrect observations of Miller and Northup on the digestive system and penetration glands.

During the summers of 1930 and 1931, Miss C. Ruth Shaw studied *C. lintoni* and carried on experiments to determine the life cycle of the species. Using the methods described by Stunkard (1930*a*), large numbers of normally emerged cercariae were available every day. In her report Shaw (1933) noted that, "Very rarely one was found that had encysted on the side of the dish, but usually they showed gradual decrease in activity unto the point of death." She confirmed Miller and Northup's account of activity and movements of the larvae, and Africa's description of the excretory system. She recognized the two pairs of cephalic glands described by previous workers, and in addition

six pairs of glands in the region just posterior to the acetabulum. For life history experiments, various marine invertebrates were placed in glass bowls with normally emerged larvae. The cercariae penetrated into and encysted in the body wall of *Nereis virens*, *Hydroides sp.*, *Lumbrinereis hebes*, *Scoloplos robustus*, *Arabella opalina*, and *Bdelloura candida*. The rich blood and oxygen supply in the parapodia of *Nereis* gave especially favorable conditions for the development of the encysted larvae, whereas she found, "In no other host did development occur; on the contrary the larvae died and the cysts became brown and opaque, according to numerous examinations." Shaw also recorded the method of penetration, and the course of development of the metacercariae in the parapodia of *Nereis virens*. Since the organs become larger and more evident as the metacercariae increase in age and size, she was able to distinguish the form of the digestive tract and the relations of the reproductive ducts. Attempts to infect the final host and complete the life history were not successful.

Following the report by Miss Shaw, the experiments were continued during the summer of 1933 in an attempt to complete the life cycle and secure the sexually mature stage of the parasite. Since *Nereis virens* had proved to be a suitable intermediate host, specimens were placed in aquaria with snails from which large numbers of *C. lintoni* were emerging, and the larvae readily encysted in the parapodia and body wall. At the end of two weeks some of the worms had thousands of cysts in the parapodia. Since Palombi (1930) had studied the life history of a similar larva at Naples and found that it developed into *Diphterostomum brusinae*, a fish parasite belonging to the family Zoögonidae, it seemed probable that the adult stage of *C. lintoni* also occurs in the intestine of marine fishes. Accordingly, when the *Nereis* were exposed to infection, specimens of various fishes which frequent the areas where *Nassa* and *Nereis* are abundant were collected and maintained in aquaria. When the fishes had been isolated for three weeks and when the metacercariae had undergone development for the same period in the parapodia of *Nereis*, the infected worms were fed to the fishes. This method permitted development of flukes acquired by the fishes before their capture, and therefore made it possible to distinguish between the experimental infection and any previously acquired one. Fishes were dissected twenty-four hours after feeding, when the recently excysted metacercariae could be easily recognized. By feeding numbers of fishes and killing them at short intervals it was possible to trace the development of the parasite in the final host, and worms with embryos in the uterus were recovered four weeks after the metacercariae were ingested. The metacercariae

excysted and young worms persisted for one to three weeks in the intestine of *Paralichthys dentatus* and *Tautoga onitis* but sexually mature specimens were not obtained. Gravid worms were secured from the intestine of *Anguilla chrysypa* and *Opsanus tau*. Attempts to infect *Tautogolabrus adspersus*, *Spheroides maculatus*, *Prionotus carolinus* and *P. strigatus* were unsuccessful. These results were presented in a preliminary report, Stunkard (1933), but since the number of metacercariae fed was enormous and the number of sexually mature worms recovered was small, and since no natural infection was discovered, the experiments have been continued in succeeding years.

Meanwhile Glading (1935) reported the presence of *C. lintoni* in *Ilyanassa obsoleta* from San Francisco Bay, where it had been introduced with the eastern oyster. According to Glading, the sporocysts occur in the liver of the snail, the tailless larvae emerge and encyst in various polychaete annelids; the list included *Capitella sp.*, *Eteone sp.*, *Streblospio sp.*, and *Nereis sp.* These encysted stages he termed "mesocercariae," and he stated that when they were eaten by crabs of the genus *Hemigrapsus*, the mesocercariae excysted, penetrated the intestinal wall and reencysted on the outer surface of the pericardium and in the muscles as metacercariae. When such encysted metacercariae were fed to the gull, *Larus californicus*, they excysted in the duodenum and developed to maturity. Adult worms were recovered near the openings of the ceca and identified as members of the genus *Levinseniella*. Accordingly, the species *C. lintoni* was transferred to the genus *Levinseniella* as *L. lintoni*. Glading declared that the validity of the life cycle as reported was proved by experimental evidence, but the evidence was not presented nor were the control measures stated.

Observations on the life cycle of *Zoögonus mirus* were reported by Timon-David (1933, 1934, 1936). This author described metacercariae, which he identified as larvae of *Z. mirus*, encysted in the muscles of Aristotle's lantern of various sea urchins from the Gulf of Marseille and along the coast of Roussillon. The parasite was abundant in *Paracentrotus lividus* and *Sphaerechinus granularis* and rare in *Arbacia aequituberculata*. Timon-David (1934) gave measurements of the cyst, of the metacercaria, and of its suckers and gonads. Concerning the life cycle he stated, "La contamination expérimentale d'un *Labrus merula* par ingestion d'Oursins parasités est extrêmement difficile à réaliser et présente de très grandes difficultés techniques. Des expériences en cours permettront peut-être une réussite. J'ajoute que j'ai observé fréquemment dans la nature des débris d'Oursins dans

le tube digestif de *Labrus merula*." He (1936) reported experiments in which metacercariae were introduced into the pharynx of three fishes, *Blennius gattorugine*. The fishes, sacrificed at the end of 45 days, yielded a dozen sexually immature worms which agreed with the descriptions of *Z. mirus*. Other control specimens of *B. gattorugine* were free of infection.

The metacercariae described by Timon-David were slightly larger than specimens I have taken from *Nereis virens*. The suckers were larger and the gonads were smaller. In my experiments it has been impossible to infect fishes by placing material in the alimentary tract. In every instance food introduced into the pharynx, esophagus or stomach was regurgitated when the fishes were returned to water. Regardless of the feeding experiments of Timon-David, however, the specimens described by him are very similar and may be specifically identical with those I have obtained at Woods Hole.

As stated previously, the work with *C. lintoni* has been continued each summer since 1930 at the Marine Biological Laboratory and it is now possible to present more complete data on the life cycle. Sexually mature worms have been reared in experimental animals and natural infections have been discovered. In abstracts Stunkard (1936*b*, 1936*c*) recorded additional experiments, the results of which confirmed his earlier report. Sufficient material has been assembled to permit a more complete study of the adult which proves to be a member of the genus *Zoögonus*, identified as *Z. rubellus* (Olsson, 1868). The assignment of these specimens to *Z. rubellus* necessitates an inquiry into the status of that species.

The genus *Zoögonus* was erected by Looss (1901) to contain a species described by him as *Z. mirus*. The specific description was based on two specimens found in the terminal part of the intestine of *Labrus merula* collected in the harbor of Trieste. The worms measured 1.55 mm. in length and about 0.45 mm. in greatest width. A conspicuous feature of these flukes was the rudimentary condition of the vitellaria and the absence of shells on the eggs. The miracidia were enclosed in thin, flexible membranes and became ciliated in the uterus.

The following year Odhner (1902) redescribed *Distomum rubellum* Olsson, 1868 and transferred the species to *Zoögonus*. Like Olsson, he found the parasites in *Labrus berggylta* (syn. *L. maculatus*) from the west coast of Sweden, but the examination of twenty fishes at the Zoölogical Station of Kristineberg disclosed only two infections and very few worms were recovered. The specimens were yellowish in

color, and situated among the folds of the hind gut. According to Odhner the body length varied from 1.1–1.4 mm. with 0.45 mm. as the greatest width. He distinguished *Z. rubellus* from *Z. mirus* on the size of suckers and length of miracidia.

The description of *Z. mirus* by Looss called attention to the fact that in this species the larvae develop to ciliated miracidia in the uterus and that the eggs are devoid of shells. The latter feature indicated that *Z. mirus* would provide favorable material for the study of gametogenesis, fertilization, cleavage, and early larval development of the digenetic trematodes. Accordingly, a number of investigators undertook the problem and in subsequent years there appeared a series of papers including the classical researches of Goldschmidt (1905), A. and K. E. Schreiner (1908), Grégoire (1909) and Wassermann (1913). In an earlier paper, Goldschmidt (1902) compared specimens collected from *Labrus merula* at Trieste with the descriptions of *Z. mirus* and *Z. rubellus* as given by Looss and Odhner and pointed out that there were no morphological differences. With ample material at his disposal he gave a more complete description of *Z. mirus*, especially of the digestive and reproductive systems. His specimens were undoubtedly specifically identical with those of Looss and the only difference between them and the account of Odhner was in the absence of yellow pigment. He was unwilling to recognize a specific distinction on this feature and pointed out that the dimensions of suckers and miracidia, characters used by Odhner to separate the species, were variable and could not serve as valid specific criteria. In the final report of his researches, Goldschmidt (1905) reaffirmed the opinion expressed in his earlier paper.

In a study of the entozoa of British marine fishes, Nicoll (1909) reported *Zoögonus rubellus* as present abundantly in every specimen of *Anarhichas lupus* from St. Andrews Bay which he examined. The worms were pale yellow in color, 0.75–1 mm. in length, and about one-half as wide as long. Nicoll referred to the descriptions of Looss, Odhner, and Goldschmidt and stated, "My specimens agree best with Goldschmidt's description." It is significant that the worms studied by Nicoll and Odhner came from the same region, whereas Goldschmidt's material was collected in the Mediterranean and presumably was identical with that of Looss. Concerning specific determination, Nicoll expressed the opinion that, "Looss's *Zoögonus mirus* is in all probability identical with Odhner's *Z. rubellus*, . . . at any rate, features sufficient to distinguish them are not at present apparent."

In a systematic review of the family Zoögonidae, Odhner (1911) maintained that the specimens of *Zoögonus* from the Mediterranean

and North Sea are specifically distinct. After collecting and comparing material from both locations, he formulated diagnoses for *Z. mirus* and *Z. rubellus*. According to his statement, in *Z. mirus*, "Die länge ausgestreckter Exemplare übersteigt nie 0.6 mm bei einer Breite von 0.18–0.2 mm." In *Z. rubellus* he found, "Länge ausgestreckter Exemplare 0.9–1.2 mm bei einer Breite von etwa 0.25 mm." He stated that the pharynx and miracidia are approximately the same size in the two species but appear to be larger in the smaller specimens of *Z. mirus*.

The Life Cycle of Zoögonus rubellus

During the years 1930–1937 inclusive, over ten thousand specimens of *Nassa obsoleta* from many different localities in the Woods Hole region have been examined for larval trematodes. *Cercariaeum lintoni* has been recovered from every area surveyed, although it was more abundant in certain places than in others. The heaviest infestation was found in snails from Sheep Pen Cove, a sheltered inlet on the northeast side of Nonamesset Island, which opens into Vineyard Sound. The snails on the Sippiwisset side of Buzzards Bay were only slightly infected; sometimes more than five hundred were examined before an infection was encountered. In general, the method employed was to collect 250 snails at a time and isolate them for four days, 10 in a finger bowl, to discover the specimens from which larvae were emerging. Each day the dishes were examined for cercariae and the snails were removed to clean bowls containing fresh sea water. When a finger bowl disclosed an infection, the snails which it contained were isolated separately to find the specimen or specimens which were liberating cercariae. The method obviously does not reveal the total infestation, since not all infected snails are giving off cercariae. Occasionally by crushing and examining snails from which mature larvae were not emerging, one was found which contained sporocysts and immature cercariae. As pointed out in earlier papers (Stunkard, 1930*b*, 1936*a*) such larvae are not infective and so are of little value for experimental purposes, although frequently structural details can be observed best in young cercariae, before the penetration and cystogenous cells are filled with secretory products. By the isolation method an average incidence of infection of about 4 per cent was found for all the snails collected, although much variation was noted. Often two collections from the same locality, taken a few days apart, gave very divergent results. In one collection only three or four infected snails would be found, whereas in the next there might be twenty or thirty. The infected snails were kept in sea water and provided larvae for study and for infection experiments.

The larvae are tailless and unable to swim. They emerge from the snails during both day and night, and temporarily appear in large numbers if the temperature of the water is raised. The cercariae become attached by their posterior ends to the bottom of the dish or to bits of debris on the substratum and constantly extend and retract their bodies. When extended they are four to five times as long as when contracted; the body is narrow and almost round in cross-section. Often it is twisted during extension. The larvae creep about on the bottom by alternately attaching the opposite ends of the body and they are also carried about by currents in the water which move them or the particles to which they adhere. Only rarely do they encyst in the water and ordinarily they die after 24 to 48 hours. The effect of dilution of sea water on the activity and longevity of these cercariae was reported by Stunkard and Shaw (1931). The presence of penetration glands well developed and the failure of the larvae to encyst in sea water indicated clearly the necessity of a second intermediate or transfer host in the life cycle. The structure and habits of the larvae made it manifest from the outset that the second intermediate host must be a bottom-dwelling species, and one which either crawls along or is sedentary with currents of water passing over it. These considerations suggested that this host should be sought among the polychaete annelids. Since the tubicolous species would not be readily infected or readily eaten by the final host, and since *Nereis virens* is prevalent on the sand and mud flats where *Nassa obsoleta* abounds, this polychaete was selected as a likely subject. The experiments with this species were uniformly successful; although the larvae penetrated into and encysted in other annelids, the best results were obtained with *N. virens* (Shaw, 1933). *Nereis* lives in burrows and the movements of the parapodia create a current of water through the tunnel. The current of water draws in small particles and the cercariae which then penetrate the parapodia. The larvae will attack the worms in the open water of a small aquarium, but it appears that they have a much better opportunity of accomplishing infection when the worm is confined within the narrow compass of the burrow. Instances of natural infection are common and frequently the worms were lightly infected when they were collected. The metacercariae were found also in specimens of *N. virens* purchased from the Supply Department of the Marine Biological Laboratory for dissection in the course in general biology at New York University.

Since *N. virens* had proved to be a suitable host for the metacercarial stage, and since the worms are easily maintained in the laboratory, this species was used exclusively for the later infection experi-

ments. Other polychaetes will probably serve as secondary intermediate hosts of the parasite, and Glading (1935) experimentally infected representatives of four different genera. For infection of *N. virens*, large flat dishes half-filled with sand and mud were arranged in series with sea water running through them. Five specimens of *N. obsoleta*, from which larvae were emerging, and ten large specimens of *N. virens* were placed in each dish. At the end of two to four weeks the larvae had encysted in great numbers and each worm contained 1,000–5,000 cysts.

Since infection of *N. obsoleta* with the asexual stages is frequent and widely distributed, it follows that some common and abundant fish harbors the sexual stage. However, many of the common fishes of shallow water were used in experiments to determine whether they would serve as final hosts and provide the sexually mature stage of the parasite. Various species were collected, maintained in the laboratory for three to four weeks, and then fed specimens of *N. virens* which had been exposed for approximately the same time to the larvae from several infected snails. Great difficulty was experienced in maintaining the fishes in small tanks and many of them would not feed. Even though the fishes had been kept in captivity for some time and should have become accustomed to the new surroundings, and although they were probably hungry, it was often very difficult to induce certain of them to eat the *Nereis*. Cunners and *Fundulus* fed readily, but most other species were refractory. Attempts to force-feed the fishes were unsuccessful and worms pushed down their throats were regurgitated. To be sure that a particular fish had eaten an infected worm it was necessary to isolate each fish and watch it until it ate the worm. So worms were suspended by fine thread and allowed to wriggle about in the water near the fish or one was dropped into the aquarium and watched for an hour or two. If at the end of that time it had not been eaten it was picked up and returned to the stock supply. Each day the attempts were repeated. On one occasion when a worm was placed near the head of a flounder, the fish opened its mouth with a yawn-like gape and the *Nereis* swam in the mouth and disappeared, although the fish made no apparent swallowing movement. This fish was dissected four days later and many immature specimens of the parasite were recovered from the intestine. Since the work was done only in the summer months and three or four weeks were required to obtain a large number of experimentally infected *Nereis*, the time left for infection experiments on fish was necessarily short. The list of fishes tested is not large and this portion of the work is not at all complete.

Nicoll found every specimen of *Anarhichas lupus* examined at St. Andrews infected with *Zoögonus*, but this fish is rare and could not be the natural host of the parasite in the Woods Hole region. Since all other previously reported hosts are members of the family Labridae, attempts were made to infect the tautog, *Tautoga onitis*, and the cunner, *Tautogolabrus adspersus*, which are the only members of the family that occur abundantly in the region. Tautogs were infected experimentally but it was difficult to maintain the fishes in our tanks and sexually mature worms were not obtained from them. The cunner is very abundant and from ecological considerations appeared to be the probable natural host. More than fifty cunners were fed infected *Nereis* and although the larvae excysted, the parasites were not recovered from these fishes later than two days after feeding. The sea robins and puffers also either failed to eat the metacercariae or they were lost from the intestine without developing. The parasites persisted for two to three weeks in the intestine of *Paralichthys dentatus* and *Tautoga onitis*, and it is probable that they may reach maturity in these species. The infections lasted throughout the summer in *Anguilla chrysypa* and *Opsanus tau* and gravid specimens were recovered. All stages of development from metacercariae to gravid worms were obtained in experimental infections. Natural infections were discovered in eels and developmental stages identical with those found in experimentally infected fishes were found together with mature specimens. From this material it was possible to identify the specimens with certainty, and their allocation to *Zoögonus rubellus* was announced (Stunkard, 1936*b*, 1936*c*).

The results obtained on fishes in captivity may not entirely parallel those which occur in nature. It is probable that fishes other than eels serve as natural hosts in the Woods Hole region but the feeding experiments gave best results with eels. Eels are common in the areas where the *Nassa* are heavily infected and undoubtedly serve as one of the hosts of the parasite. Examination of eels during successive years has shown a heavier infection in early summer. Our records have been supplemented by data collected by Dr. A. V. Hunninen, who dissected eels during two seasons while working on another problem. Grateful acknowledgment is made to Dr. Hunninen for permission to publish the results of his collection of *Z. rubellus*. Ordinarily the number in a host is one to five, although in one instance 56, and in another 165 worms were removed from a single intestine. Both of these eels were from the Eel Pond. In a series of dissections done in June, 1937, 30 eels were examined. Twenty of them were infected and yielded a total of 339 worms. In another series done in July and August, 1936, the

dissection of 100 eels provided only 10 infections and 46 worms. Twenty-seven eels were dissected during the first two weeks of September, 1936, and all were negative. Dr. Hunninen examined 72 eels during the summer of 1937. He found 33 infections and 439 worms, although most of the parasites were collected in June and July.

Since the metacercariae may undergo considerable development in the second intermediate host, it appeared likely that such development was necessary before they could be established in the definitive host, but later observations indicate that the metacercariae are infective very soon if not immediately after encystment. When a toadfish or eel is dissected two days after it has eaten a *Nereis* that had been exposed to infective cercariae for several weeks, one finds a series of young worms. Some of them are only slightly larger than the cercariae and it is apparent that they had only recently encysted and had not undergone development in the worm. It appears therefore that young, recently encysted metacercariae are infective for fishes but that they require a much longer time to reach maturity than metacercariae which have undergone maximal development in *Nereis*. The largest metacercariae require about eight weeks to reach maturity in the final host.

Experimental infection of snails has been produced and, although the time from infection of the snail until the emergence of mature cercariae is unknown, our experiments indicate that more than ten weeks are required. For completion of the life cycle, therefore, about six months are required, although the time varies with the season and inversely as the temperature.

Stages in the Life Cycle of Zoögonus rubellus

The Adult

Living specimens are very mobile, especially the preacetabular portion; a worm may extend to 1.55 mm. and contract to one-third that length. The width varies inversely as the length and measures from 0.09–0.3 mm. Apart from the development of the reproductive organs and the resulting increase in size, the worms are very little larger than fully grown metacercariae.

Fixed and stained specimens (Fig. 7) measure 0.7–1.2 mm. in length and 0.18–0.28 mm. in width. When alive they may be colorless or tinged a greenish yellow. The yellow material is dispersed in the parenchyma, although frequently the contents of the intestine are more yellow than the tissues and it appears that the color is dependent upon the food of the worm. The cuticula is beset with spines, although

they gradually diminish in size and number posteriorly and may be absent on the terminal part of the body. The acetabulum is usually situated about one-fourth of the body length from the anterior end, although this distance varies from one-third to one-sixth. Ordinarily the acetabulum is slightly larger than the oral sucker although both are smaller when contracted than when extended and flattened. In normal worms the acetabulum measures 0.075–0.085 mm. in diameter and in pressed specimens it may expand to a width of 0.1 mm. The oral sucker of mature specimens is 0.07–0.08 mm. in diameter. It is followed by a long prepharynx which is spanned dorsally by the commissure of the central nervous system. The pharynx is spherical, oval to pyriform, usually wider posteriorly, and measures 0.04–0.06 mm. in diameter. Typically it lies a short distance anterior to the acetabulum but the position is very inconstant. The esophagus is long, the bifurcation of the digestive tract ordinarily occurring behind the level of the testes. As noted by Nicoll, the posterior end of the esophagus is often expanded and the ceca arise from the enlargement. The short ceca are either saccate or tubiform and end blindly at or near the ovarian level. Goldschmidt observed that only the fundus of each cecum bears epithelium.

The excretory system of the adult is unchanged from that in the cercarial stage. It is represented by the flame cell formula $2(2 + 2) + (2 + 2)$.

The testes are lateral, situated in the zone immediately posterior to the acetabulum, the testis of the poral side displaced slightly posterior to the other. They are spherical to oval and may be longer in either axis depending on the extension or contraction of the specimen. They measure from 0.032–0.072 mm. in diameter. Vasa efferentia arise from the medial faces of the testes and unite to form a duct, which enters the cirrus sac, where it expands to form a large bipartite, S-shaped seminal vesicle. The vesicle is continued by a narrow duct, surrounded by glandular cells which discharge into it, which communicates with the cirrus. The latter organ is eversible and covered with fine spines, although the spines are often indistinguishable in fixed and stained specimens. The clavate cirrus sac (Fig. 5) is bent laterally, and extends from the region between the testes to the genital pore. The longitudinal muscles of the wall are strongly developed and it may overlie the posterior portion of the acetabulum. The pore, surrounded by conspicuous radial and circular muscles, is lateral, near the level of the posterior margin of the ventral sucker, with the male opening dorsal and anterior to that of the metraterm.

The ovary is spherical to oval, 0.04–0.064 mm. in diameter. It is located between the ends of the digestive ceca, usually slightly lateral in position. The oviduct arises at the median side and turns posteriad where it receives a short duct from the seminal receptacle and where Laurer's canal emerges to pass to the dorsal surface. A small, compact vitelline mass is located beside the ovary and a short duct leads from it to the oötype. The initial portion of the uterus passes forward and coils about. The uterus then passes backward almost to the posterior end of the body, returns to the zone of the digestive ceca, passes backward a second time to the region of the excretory bladder and then runs forward to the genital pore. The loops of the uterus follow a winding course and their position shifts with the extension and retraction of the postacetabular portion of the worm. The portion of the vitelline cells concerned with shell production is absent or inoperative, since the larvae are enclosed in a membranous sac without a shell. When the worms were placed in sea water diluted one-half with distilled water, flattened vacuoles appeared around the larvae and the cilia of the six to ten oldest miracidia beat regularly. The development of the larvae in the uterus was described by Goldschmidt (1905).

The Miracidium

The miracidium (Fig. 8) is ovate, somewhat pointed anteriorly. The body wall consists of four rows of ciliated epidermal cells. The number was not determined precisely and there is apparently some variation in the anterior two rows. As the larvae develop they consist of cell masses which stain deeply but mature miracidia do not stain well and when fully differentiated only a few of the nuclei retain the stain. The anterior tip is very mobile and it may be poked about in exploratory-like movements. There are two large glandular cells which extend more than half of the length of the larva and open at the anterior end. Their contents are finely granular. Immediately behind these glandular cells there is a cluster, usually four to eight, of large cells with deeply staining nuclei. They are undoubtedly the germinal cells which produce the next generation of larvae. There are two flame cells, one situated in the anterior, the other in the posterior portion of the miracidium. Their ducts were not traced to the excretory pores. Living miracidia in their sacs measured 0.1–0.13 mm. by 0.045–0.056 mm. They may become longer and thinner or shorter and thicker. Miracidia in the uterus of fixed and stained worms measure 0.09–0.12 mm. by 0.036–0.045 mm.

When mature worms are removed from the intestine of the eel, the miracidia may be extruded while within their membranous coverings.

The anterior end of the larva emerged first in all specimens observed. Such larvae may escape from their sacs in a short time, and they swim actively in a spiral course. In swimming the body is elongated, and the miracidia swim so fast that it is exceedingly difficult to follow a larva under a binocular microscope. Since the miracidia normally become free-swimming, it is apparent that they meet specimens of *N. obsoleta* and bore into them. Actual penetration was not observed, but the number of swimming larvae was small and the probability of observing one until it found and entered a snail is very remote. It is probable that under natural conditions the miracidia enter the siphon of the snail with the current of water and then pierce the body from some point on the wall of the branchial cavity.

The Sporocyst Generations

The sporocysts which produce cercariae were described by Linton (1915) and Shaw (1933). They are very numerous and are distributed throughout the lymph spaces of the snail, chiefly near the body wall and in the interlobular areas of the digestive gland and gonad. They may be present in different and widely separated locations in the same host and, since they are held together in masses by the connective tissue of the snail and are unable to move about, their distribution indicates that at an earlier period these sporocysts or their progenitor had migrated to the location in which they were found. They are thin-walled oval sacs with a birth pore at one end. At the end opposite the pore a mass of germinal cells and germ balls is followed by progressive development of the larvae until fully formed cercariae are present near the pore. The number of larvae in a sporocyst is variable and apparently depends on its age and condition. In young and vigorous sporocysts there may be 30–40 developing larvae while in older ones there may be only two or three immature cercariae and no germ balls. In snails which had been kept in the laboratory with little food for two or three months and from which cercariae had emerged during all that period, the tissues of the host were emaciated and many of the sporocysts were empty. This observation suggests that the germinal tissues had become exhausted. The cercariae normally leave the sporocysts and complete their development in the lymph spaces of the snail. In the examination of crushed snails, it is frequently impossible to determine whether the free cercariae were liberated from sporocysts in the crushing process or whether they had emerged previously. Study of snails cut in serial sections, however, shows many immature cercariae free in the lymph spaces of the host. Although they may leave the

snail by other routes, the examination of many snails, both by dissection and serial section, indicates that the cercariae pass by way of the vascular system to the branchial region, and that when fully mature they break out into the mantle cavity and emerge through the excurrent siphon.

In snails from which cercariae were emerging, all of the sporocysts appear to be producing cercariae. While the germ ball stages of all generations are similar and positive determination may occasionally be impossible, in no case have sporocysts which contain unmistakable daughter sporocysts been found in snails from which cercariae were being liberated. Both daughter sporocysts and cercariae have never been observed in the same sporocyst and it is almost certain that the generations which produce sporocysts are distinct from the one which produces cercariae. Rediae do not occur in the life cycle.

An attempt was made to secure early developmental stages of the parasite in the snail. Fifty small specimens of *N. obsoleta* were collected early in June, 1937, from an area where infection by *C. lintoni* is rare. Forty-eight of them were dissected and proved uninfected; the remaining two were exposed on July 20th to eight miracidia of *Z. rubellus*. No trematode larvae emerged from these snails during the summer and they were killed and fixed entire on September 14th.

EXPLANATION OF PLATES

Abbreviations

cg	gland cell of miracidium	*ic*	intestinal cecum
cs	cirrus sac	*lc*	Laurer's canal
dm	developing miracidium	*mm*	mature miracidium
ev	excretory vesicle	*ov*	ovary
fc	flame cell	*sr*	seminal receptacle
gb	germ ball	*st*	stylet
gc	germ cell	*ts*	testis
gd	ducts of penetration glands	*vt*	vitellaria
gm	degenerating miracidium		

PLATE I

FIG. 1. *Z. mirus*, dorsal view, after Goldschmidt (1902).

FIG. 2. *Z. mirus*, ventral view, after Looss (1901).

FIG. 3. *Z. rubellus*, ventral view, after Odhner (1902).

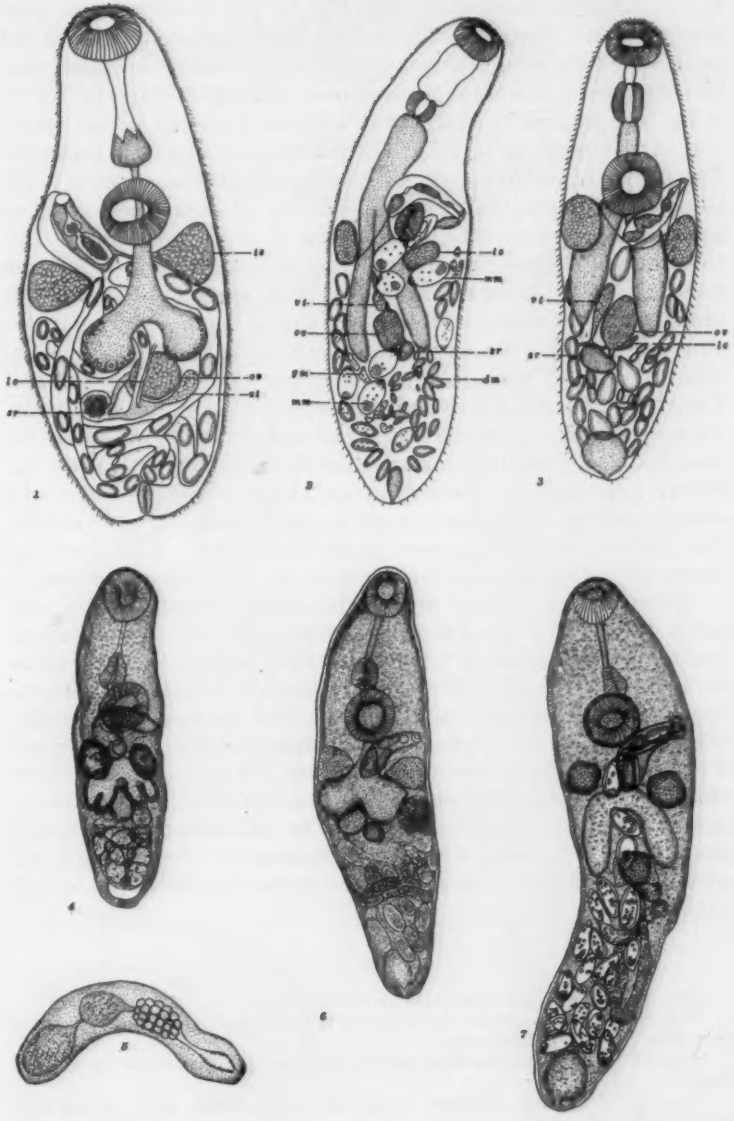
FIG. 4. *Z. rubellus*, ventral view, camera lucida tracing of specimen from toadfish, three weeks experimental infection.

FIG. 5. *Z. rubellus*, free-hand sketch of cirrus sac.

FIG. 6. *Z. rubellus*, ventral view, camera lucida tracing of specimen from eel, seven weeks experimental infection.

FIG. 7. *Z. rubellus*, ventral view, camera lucida tracing of specimen from eel, natural infection.

PLATE I



Later they were cut in serial sections. In both of them the haemocoele spaces contained small sporocysts (Fig. 9), but no other stages were present. The infection is about three times as heavy in one of the snails as in the other. The location of the parasites shows clearly that they migrate by way of the blood sinuses, but in spite of careful search it has been impossible to recognize with certainty the mother sporocyst or sporocysts in which the dispersed generation was produced. The absence of cercaria-producing sporocysts after three months in the laboratory indicates that the snails were not infected before collection and demonstrates that the infection was an experimental one. This conclusion is afforded further support by the uniformity in appearance and stage of development of the parasite in both of the snails. Although they are widely scattered, the sporocysts are very similar in structure. Most of them are solid bodies with aggregations of germinal cells interspersed among the mesenchymal tissues of the parasite. They have irregular shapes and apparently possessed much mobility. These sporocysts frequently contain lacunae or small lumina but the condition of the germinal masses indicates that they are not the definitive generation in which cercariae are developed. In fact, the germinal masses often show secondary aggregations in their interior (Fig. 9) which probably represent another generation. In the germinal tissue, cell multiplication is rapid and mitotic figures are numerous.

The soft parts of *Nassa obsoleta* are usually embedded in the mud and it is likely that the miracidia normally enter the siphon with the incurrent water and from the branchial cavity penetrate into the tissue of the host. Although information concerning the sporocyst generations is yet too fragmentary to permit a final conclusion, a tentative interpretation of the findings in the experimentally infected snails may be made. There is strong probability that each of the miracidia which entered the snails underwent metamorphosis to form a primary sporocyst and that this generation had become unrecognizable after producing a generation of secondary sporocysts which had left the original one. The identification of the sporocysts which were widely

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PLATE II

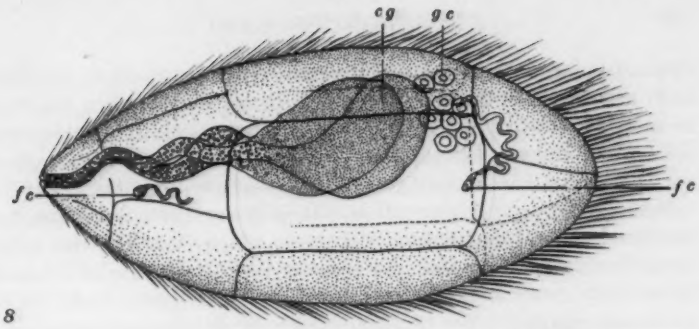
FIG. 8. *Z. rubellus*, free-hand sketch of miracidium.

FIG. 9. *Z. rubellus*, camera lucida tracing of a section of a sporocyst containing germ balls, experimental infection.

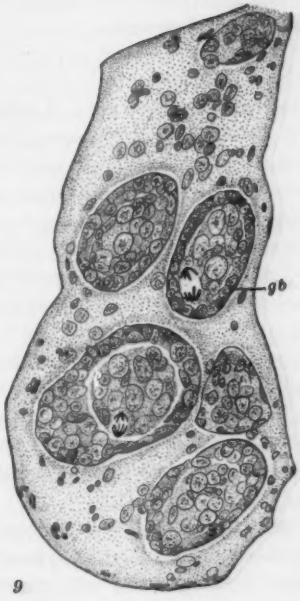
FIG. 10. *Z. rubellus*, free-hand sketch of stylet and openings of penetration glands.

FIG. 11. *Z. rubellus*, free-hand sketch of cercaria, dorsal view, stained with neutral red.

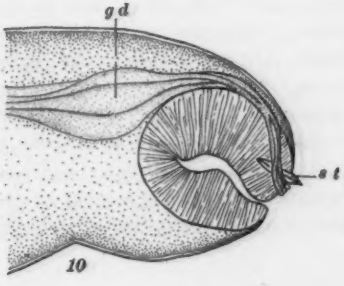
PLATE II



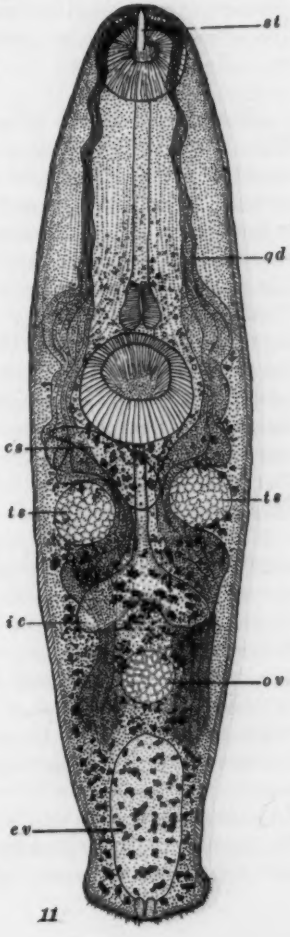
8



9



10



11

scattered nine weeks after infection is not an easy matter. It is clear from their number that they are not primary sporocysts. They may be secondary sporocysts, although their number and the time interval, July 20–September 14, which had elapsed since the infection of the snail, suggests that another generation may have intervened. If they belong to the second generation, the primary sporocyst must have produced about 100 daughter sporocysts which were liberated almost simultaneously and in a similar stage of development, with its subsequent retrogression and disappearance. The germ balls in the sporocysts do not have the appearance of developing cercariae, as observed in natural infections, and it is probable that another generation of sporocysts occurs before the production of cercariae.

The Cercaria

Descriptions of the cercaria have been given by Leidy, Linton, Miller and Northup, Africa, Shaw, and Glading. My observations concerning size, shape and movement agree with those of previous authors. The posterior end of the larva (Fig. 11) may be expanded to form a rim, and the contraction of the center of the tip produces a suction by means of which the cercaria becomes attached. The first clear and accurate account of the digestive system was made by Shaw (1933), who corrected the erroneous descriptions of Miller and Northup and of Africa. The study of metacercariae permitted the identification also of the reproductive organs, rudiments of which had been observed by Linton and by Miller and Northup. Linton's observations concerning the testes were confirmed, and the structures tentatively identified by him as the primordia of the ovary and uterus were shown to be those of the copulatory organs and female genital complex respectively.

The description of the excretory system given by Africa was confirmed by Shaw although the details had been worked out previously as noted by Stunkard (1932). The vesicle is saccate and may extend two-fifths of the distance to the acetabulum. From the sides, near the anterior end of the vesicle, collecting ducts pass anterolaterad and then bend mediad, passing forward in a sinuous course below the digestive ceca and median to the testes. On either side in the region between the testes and the acetabulum, the primary collecting duct divides into secondary anterior and posterior branches. The anterior branch continues forward, lateral to the acetabulum, and in front of the sucker it divides into tertiary anterior and posterior branches. The tertiary anterior branch continues forward to the level of the posterior margin of the nerve commissure where it divides to form the

capillary tubules to the anterior pair of flame cells. The cells lie equidistant, one in front and the other behind the commissure. The anterior cell is situated ventrally, at the side of the oral sucker, below the ducts of the penetration glands. The capillary which leads to the posterior member of the anterior pair of flame cells may parallel the tertiary duct for a short distance but the cell body is usually lateral in position. The tertiary posterior branch is recurrent and runs parallel with the anterior secondary duct for a short distance. It soon divides into two capillaries, one of which leads to a flame cell situated near the anterior margin of the acetabulum and the other to a flame cell lateral in position near the level of the caudal margin of the sucker. The arrangement of the excretory structures in the posterior half of the body is the counterpart of that in the anterior half. The posterior secondary duct passes backward beside the primary one for a short distance and then divides to form anterior and posterior tertiary branches. The anterior branch is short and divides into two capillaries, one of which leads to a flame cell at or near the level of the testis and the other to a flame cell somewhat more posterior in location. The posterior branch runs backward almost to the level of the vesicle where it divides to form capillaries that supply the caudal pair of flame cells. One of these cells lies in front and the other behind the opening of the collecting duct into the vesicle.

The number and arrangement of the unicellular glands in the cercaria has proved an exceedingly difficult problem. Miller and Northup reported, "One pair of prominent larval gland ducts, with granular contents, on either side of body, mistaken by Linton for excretory vesicles; four large eosinophilic larval glands, staining also with intra-vitam neutral red, but clearly observable only in sections." In their figure two large gland cells were portrayed on each side, behind the acetabulum, with ducts which pass forward to open near the stylet. Concerning the location and nature of the "four large eosinophilic larval glands," the authors made no statement. Miller and Northup's description of the penetration glands was copied by Africa. Shaw stated, "The two pairs of cephalic glands shown in Fig. 1 have been described by previous workers. But in addition, by intra-vitam staining with neutral red, I have been able to recognize six pairs of glands in the region just posterior to the ventral sucker." The outlines of these cells were sketched in the figure by Shaw, although their function was not discussed.

For six seasons I have studied the unicellular glands of this cercaria. The parenchyma is filled with refractive spherules which interfere with observation of the gland cells. Various techniques and many vital

dyes have been used in an attempt to differentiate between cystogenous and penetration glands, and to determine the number and position of each type. The application of vital dyes to living specimens has given better results than the staining of fixed specimens. In young cercariae, removed from sporocysts, the gland cells are not recognizable. As the larvae mature the secretion appears in the form of fine granules, but does not stain. The cells are small, difficult to observe, and the two types are morphologically identical. Later, in penetration glands, the granules increase in size and pass into the ducts, where they are stainable. The two pairs of cephalic or penetration glands described by previous authors are merely enlargements in the ducts of glands, whose cell bodies lie farther posteriad.

Instead of two pairs of penetration glands, there are certainly five and probably six pairs of them. The bodies of these cells lie in the region behind the digestive ceca and on either side the ducts intertwine with each other and with the principal excretory tubules to form a bundle which leads forward. From the level of the digestive ceca to that of the oral sucker, the ducts of the penetration glands parallel those of the excretory system and this association increases the difficulty of counting them. The course of the ducts has been noted in the description of the excretory system and is represented in Fig. 11. At the anterior end, the ducts pass over the sucker on either side (Fig. 10) and open ventral to the tip of the stylet.

When the cercariae become active, the contents of the penetration glands pass forward filling the ducts, and the cell bodies are relatively inconspicuous. The cell bodies are lobed, irregular in size, changeable in shape, and often superimposed in such a way that it is impossible to resolve the cell boundaries separating overlapping lobes. The nuclei are seldom clear, although occasionally some of them stand out conspicuously. One cannot be certain, however, that the nuclei of all these cells are visible at the same time and accordingly the number of penetration glands cannot be determined with certitude by counting their nuclei. The ducts of the penetration glands twist about one another and are invisible when empty, so that ordinarily it is not possible to trace a tubule very far. A duct may be much increased in diameter for a short distance and then it may disappear as it passes between other ducts. As the body of the cercaria extends and retracts, the gland ducts are bent and bowed and the secretion accumulates at particular locations; e.g., at the anterior and posterior margins of the oral sucker, acetabulum, and testes. In these regions, expanded portions of the ducts may simulate cell bodies and have undoubtedly misled earlier observers. Occasionally clear spherical areas may

present the appearance of nuclei, but their size is inconstant and they are probably vacuoles since they may pass along the ducts and sometimes they disappear or break up into smaller spherules. The secretion is usually clear when extruded and persisting granules soon liquefy.

Cystogenous glands have not been reported previously. These cells are scattered over the body from the level of the oral sucker to the caudal end. Twenty-six of them have been counted in front of the acetabulum. There is a cluster behind the acetabulum and they extend into the region of the penetration glands. At least sixteen are present in the area around the excretory vesicle. The positions and shapes of the cells change with movements of the worm and it has been impossible to trace the ducts of these glands. The cells measure about 0.012 mm., and the nuclei about 0.006 mm. in diameter. By the use of vital dyes it is sometimes possible to distinguish slight differences in form, size, and color between the cytoplasmic inclusions of cystogenous and penetration glands. When treated with dilute neutral red, cystogenous cells may have flaky, reddish granules and penetration glands more spherical and somewhat violet-colored granules. But the form of the inclusions is not constant and, like the staining reaction, probably differs in various stages of the secretory cycle. Similar changes in color reactions of penetration glands in schistosome cercariae were discussed by Stunkard (1930*a*). In *C. lintoni*, the position of certain gland cells may indicate their nature, but in the region of the digestive ceca it is frequently impossible to distinguish between cystogenous and penetration gland cells.

The Metacercaria

The metacercarial stage was first secured experimentally and described by Shaw (1933). Later accounts have added little to her report. In the second intermediate host the cercarial structures are resorbed or lost and development is continued. Since the larvae develop in the annelid, it serves as a true intermediate rather than a mere transfer host, and this fact is probably correlated with a certain degree of specific relationship between host and parasite. Whether the larvae will infect sea urchins at Woods Hole remains to be discovered.

Development in the Final Host

Although the metacercariae excyst in the intestine of several species of fish, mature specimens have been recovered only from the toadfish and the eel. In abnormal hosts the worms are soon lost and even in natural infections the number of parasites recovered is usually small. Natural infections at Woods Hole have been found only in

eels, although laboratory experiments indicate that the toadfish may serve as a final host.

The length of time required for the attainment of sexual maturity depends on the degree of development attained by the parasite in the second intermediate host. In a metacercaria which has reached maximal size, the anterior portion of the body and the suckers are almost as large as in the adult condition. The development in the final host therefore consists almost entirely in the maturing of the reproductive organs. The male system develops first and spermatozoa were observed in the seminal vesicle of specimens removed after one week in the intestine of a toadfish. At that time the prostate cells are active and the cirrus is spined. Fertilized ova produce germ balls which increase in size and differentiate into miracidia in the uterus of the worm. As noted previously, there is no shell and the larvae are enclosed by thin, flexible, membranous coverings. Fully formed, ciliated miracidia were obtained seven weeks after fishes had ingested mature metacercariae. If the metacercariae are less well developed, a correspondingly longer time is required to reach maturity. As the worm becomes gravid, the postacetabular region of the body increases in size. Specimens recovered after three weeks and seven weeks in the intestine of the final host are depicted in Figs. 4 and 6 respectively. A fully mature worm from a natural infection is portrayed in Fig. 7.

Discussion

For more than thirty years there has been difference of opinion concerning the validity and specific distinctness of *Zoögonus rubellus* and *Z. mirus*. Goldschmidt (1902, 1905) found the only difference between the two forms to be one of color, and that morphologically they were indistinguishable. The same opinion was expressed by Nicoll (1909). The figures of *Zoögonus* by Looss (1901), Odhner (1902) and Goldschmidt (1902) are reproduced for comparison (Figs. 2, 3, 1). The account of Odhner (1911), maintaining the existence of two distinct species, *Z. rubellus* from the North Sea and *Z. mirus* from the Mediterranean, was based on a comparative study of specimens which he had collected from the two localities. Odhner distinguished the two species on differences in color and size, size of suckers and shape of sucker cavities, location of the acetabulum, length of digestive ceca, position of cirrus sac, and number of eggs in the uterus. The specimens of *Z. mirus* studied by Odhner were small and apparently somewhat contracted. His statement that the length of extended worms never exceeds 0.6 mm. cannot be accepted since the type specimens described by Looss measured 1.55 mm. in length. The size of suckers in *Z.*

mirus as given by Odhner does not agree with the statement of Looss, whose measurements of *Z. mirus* agree with Odhner's figures for *Z. rubellus*. It is to be remembered that Looss had only two specimens and, as noted by Nicoll, the worms were somewhat distorted which accounts for his observation that the acetabulum is slightly smaller than the oral sucker. Furthermore, in soft-bodied worms, which are capable of much extension and contraction and in which one part of the body may be retracted while another is extended, the movements of the animals cause constant variation in the shape and relative position of the organs. It is apparent from Odhner's account that his specimens from the North Sea were more extended than those from the Mediterranean, and this condition may explain the differences noted in the position of the acetabulum and cirrus sac and in the shape of the digestive ceca. The specimens from the Mediterranean were smaller, and the fact that they contained fewer larvae indicates that they were not fully mature. Indeed, the size of the body is largely influenced by the degree of sexual maturity and the worms increase in size as the number of eggs increases. Color is a variable feature and an unsatisfactory criterion for specific determination. Nicoll reported that his specimens from *Anarhichas lupus* were pale yellow, which suggests a condition intermediate between those observed by Odhner. An examination of Odhner's paper and comparison of his account with earlier descriptions left grave doubt concerning the specific distinctness of *Z. rubellus* and *Z. mirus*.

The study of specimens from the eel and toadfish at Woods Hole has provided data for a solution of the problem. Worms recovered at four-day intervals from experimentally infected fishes show a continuous series of developmental stages. Some of the specimens which have developed for seven to eight weeks in the intestine of the final host agree entirely with Odhner's diagnosis of *Z. mirus*, while older infections yielded worms which were more mature and except for the intense yellow color, agree with Odhner's description of *Z. rubellus*. Some of the present specimens were colorless, while others had a faint greenish-yellow color. Since Odhner's diagnosis of *Z. mirus* does not agree with Looss' original description of the species, and since specimens from experimental infections show transition through a series of forms intermediate between *Z. rubellus* and *Z. mirus*, there appears to be no adequate criteria for distinguishing between them.

The work of Palombi (1930) showed that *Cercariaeum crispata* Pelseneer is the larval form of *Diphterostomum brusinae* Stossich, a member of the family Zoögonidae. In that paper Palombi pointed out that *C. lintoni* is very similar to the larva of *D. brusinae* and suggested

that it might also be a member of the same family. Palombi found that the life cycle of *D. brusinae* requires about six months for completion and he questioned the explanation offered by Miller and Northup that "semi-annual visitation of Woods Hole by the definitive vertebrate hosts" might account for the two periods of maximal infection of snails in June and December. He found similar high incidence of infection in mollusks in August and February and attributed this fluctuation to the developmental cycle of the parasite. The results of the present study confirm in large part the postulates of Palombi.

The report of Glading (1935) that *C. lintoni* is the larval form of a species of *Levinseniella* may be explained on the assumption that the crabs to which he fed infected polychaete annelids were already infected with metacercariae of the latter genus and that they developed to maturity in the gull, *Larus californicus*. Indeed, Lebour (1911) and more recently Young (1938) have shown that the metacercariae of *Levinseniella* and related forms do occur in crabs.

Discussion of the life history of *Z. rubellus* requires consideration of *Cercariaeum reticulatum*, a species described by Stunkard (1932) from *Nassa reticulata* at Roscoff, France. The original description is brief and incomplete in many respects. This larva is very similar to *C. lintoni* and they are certainly closely related. In fact, review of notes and sketches made at Roscoff indicates that they may be identical. The discovery of the life cycle of *C. lintoni* opens the way for an experimental attack on the problem.

The group *Cercariaeum* Diesing, 1855 has been used to contain a number of little known and doubtful forms. It was restricted by Lühe (1909) to distome larvae without caudal appendages, which develop in rediae or simple sporocysts. Palombi (1930) showed that *Cercariaeum crispata* Pelseneer is the larval stage of *Diphterostomum brusinae*, a member of the family Zoögonidae. Stunkard (1934) reported the life cycle of *Typhlocoelum cymbium*, a member of the family Cyclocoelidae, and showed that the larva is a *Cercariaeum*. In that paper he stated that since members of *Cercariaeum* develop into such widely different adults as *D. brusinae* and *T. cymbium*, it is apparent that the group is an assemblage of unrelated species and has no genetic unity. The discovery of the life history of *Z. rubellus* confirms that opinion.

The larvae of digenetic trematodes have various types of caudal appendages and it is not surprising that in certain of them such structures should be entirely lacking. The term *Cercaria* may be applied with equal propriety to all of these larvae, and the name *Cercariaeum* should be suppressed.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

JULY 5, 1938

Endocrine activity of the placenta in mice. W. H. Newton. (The published paper will appear in an early number of the *Anatomical Record.*)

Evidence for hormonal control of uterine motility by the hypophysis in the rabbit. J. K. W. Ferguson and H. O. Haterius.

By inserting fine bipolar electrodes into the brain of a rabbit it is possible to apply electrical stimulation to the region of the pituitary stalk. When this is done in rabbits two to eighty hours after parturition while recording the motility of one horn of the uterus, an enormous increase in the frequency and amplitude of uterine contractions is observed after a latency of two or three minutes. This effect of electrical stimulation of the stalk is strikingly similar in form and duration to that of 0.3 units of Pitocin injected intravenously. The effect of epinephrine injected intravenously (1 cc. of 1/50,000) is very different, being chiefly an apparent inhibition. Furthermore, the response to epinephrine lasts only a few minutes while the response to Pitocin or electrical stimulation may last for nearly an hour.

The response to electrical stimulation is abolished by destruction of the pituitary stalk and posterior lobe or of the stalk alone. It is not abolished by severing all nervous connections between the brain and the uterus provided the pituitary complex and its connection with the uterus by the blood stream remains intact. After destruction of the stalk the activity of the uterus slowly but progressively diminishes. The evidence establishes to our satisfaction that a hormone capable of stimulating uterine motility can be released from the pituitary *in vivo*. It also suggests that the uterine activity of the parturient animal is directly dependent on the release of a pituitary hormone. Similar experiments on virgin rabbits have given indefinite results.

Experimental studies on the genital system of the male anuran. Roberts Rugh.

By means of micro-injection of a solution (made up of one part India ink and two parts Holtfreter's modification of amphibian Ringer's) into the vas efferens in the direction of the Anuran testis, the distribution of the seminiferous tubules can be made out. If the injection is made in the direction of the kidney, the path of spermatozoa through the kidney to the ureter can be seen. The vas efferens (of which there are 8–12 branching ducts) branches as it enters the kidney, and feeds 4 or more Malpighian corpuscles. Ink can be seen in the Malpighian corpuscle and passing out dorsalward (in the coils of the uriniferous tubules) through the kidney substance and then laterally across the kidney to the ureter (Wolffian or mesonephric duct).

If a hibernating male frog (*Rana pipiens* or *Hyla crucifer*) is injected with the anterior pituitary hormone, and the urinogenital system is studied histologically at intervals of 9–36 hours after treatment, spermatozoa may be traced from their Sertoli cells (in the seminiferous tubules), through the vasa efferentia, into the Malpighian corpuscles, through the uriniferous tubules to the ureter and ultimately to the seminal vesicle—in a manner exactly similar to the course of ink traced by the

above-mentioned technique. In *Rana pipiens* about 50 of the 2,000 Malpighian corpuscles have this dual function, and in *Hyla crucifer* about 40 of the 400 are so involved.

This study offers two major conclusions: First, the anterior pituitary hormone acts on the testis of the hibernating frog in a manner similar to its effect on the anuran ovary, i.e., it liberates the gametes. This liberation in the testis may be aided (as it is in the ovary) by muscular contraction of the gonad stroma. Second, the path of spermatozoa is from the seminiferous tubule through the vas efferens to the Malpighian corpuscle, at which point the genital ducts and urinary ducts become one and the same. There is no morphological or histological evidence of any Bidder's canal running along the mesial margin of the kidney, as described for many European Anura.

JULY 12

The rôle of chromogenic bacteria in ciliate growth. G. W. Kidder and C. A. Stuart.

It has been found that there are marked differences in the reactions of the ciliate *Colpoda steini* toward the various species of bacteria normally found in hay infusions. Sixteen types were tested and comparisons made of the ciliate reproduction in suspensions of pure strains in distilled water. The ciliates were sterilized before each test by centrifugation and dilution, and the sterility tested by broth inoculations and agar plate streaks.

It was found that the best growth and reproduction could be obtained in suspensions of *Aerobacter cloacae*. Decreasingly successful were pure suspensions of the following types: *Serratia marcescens* (non-pigmented), *Spirillum rubrum*, *Vibrio* sp., *Sarcina lutea*, *Staplococcus aureus*, *Pseudomonas* (3 species), *Rhodococcus roseus*, *Flavobacterium* (lemon yellow) and *Staphlococcus albus*. It should be noted that abnormalities occurred when the ciliates were fed on suspensions of *Rhodococcus* and that death rather than the normal encystment followed a short reproductive period in suspensions of *Staphlococcus albus*.

Another group of bacteria failed to support growth entirely. *Flavobacterium* (orange) proved to be non-toxic but entirely rejected as food by the ciliates. Pigmented *Serratia marcescens* caused death in all trophic organisms within 5 to 30 minutes, the red pigment concentrating in the cytoplasm. Ninety per cent of the cysts were likewise killed by the pigment which entered the protoplasm through the cyst wall. *Chromobacterium violaceum* affected the ciliates in a manner similar to the pigmented *Serratia* except that there were no survivals. The water-soluble pigments of the various species of *Pseudomonas* tested and of *Bacillus niger* were instantaneously fatal to all ciliates.

Because of the effects noted above and especially because of the ability of *Colpoda steini* to select particular strains of bacteria suitable as food, it is evident that in a mixed culture there will arise a preponderance of rejected bacteria which will in turn prove detrimental to the ciliates. It is therefore extremely important to sterilize completely all ciliates before attempting any critical observations on nutrition. In addition to the original sterilization, rigid bacteriological techniques are required to prevent future contaminations.

Some effects of temperature on the reproduction of Chilomonas paramecium. Jay A. Smith.

The medium used in the experiments was composed of inorganic salts plus sodium acetate treated to insure sterility at all times. The temperature varied only 0.25° C. at all temperatures, namely 35°, 30°, 27°, 23°, 18°, 14°, and 9° C.

After extended periods of time at various temperatures, the frequency of division became constant. This frequency of division was zero at 9.5° C., but the chilomonads

survived for five weeks at this temperature. As the temperature rose, the frequency of division increased to a maximum of about 0.14 divisions per hour between 26° and 30.5° C. Above 30.5° C. the frequency fell until it reached zero at 35° C.

As the chilomonads were exposed to temperatures of 14° C. and 9.5° C. for prolonged periods of time, the temperature and the length of the exposure to the temperature necessary to kill them decreased. This decrease was from 168 hours at 35° (after exposure to 14° C. for 1 week) to 48 hours at 35° C. (after exposure to 14° C. for one week followed by 9.5° C. for 3 and 4 weeks), and from 35° C. to 30° C. The latter temperature became a lethal temperature only after exposure to 14° for one week followed by 9.5° C. for three and four weeks. This decrease was probably due both to a decrease in the frequency of division and to an increase in the death rate.

After prolonged exposure to temperatures of 14° and 9.5° C. the frequency of division at higher viable temperatures was slightly lower than the normal frequency of division at these higher temperatures. This low frequency of division probably had its source in the frequency of division during the first week after transfer from the low temperatures because after extended periods of time at any viable temperature the frequency of division assumes a constant value that is reproducible at that temperature.

During the first 48 hours after transferring the chilomonads from a low viable temperature to other viable temperatures, there was a gradual increase in the frequency of division from the low value at the lower temperature to higher values at the higher temperatures. This indicates that the acclimitization necessary to allow the chilomonads to reproduce at higher temperatures is not an instantaneous acclimitization, but a gradual change, taking 48 hours or so.

Adjustment of the marine Amoeba, Flabellula mira Schaeffer, to changes in the total salt concentration of the outside medium. Dwight L. Hopkins.

This amoeba can be cultured in any concentration from sea water diluted 20 times to sea water concentrated 10 times by evaporation. It never forms contractile vacuoles which are typical for fresh water protozoa. The food vacuoles when extruded from the cell contain large quantities of water as well as fecal material. The rate of elimination of fluid material by means of the vacuoles is inversely proportional to the concentration of the medium and directly proportional to the volume of the amoeba provided that the amoeba has been adjusted to the medium and is active and feeding.

When the concentration of the medium is decreased, the amoeba swells at first and then shrinks to its original volume. During this shrinkage elimination of fluid by the vacuoles does not nearly account for the volume loss. If the concentration of the medium is increased the amoeba shrinks at first and then swells to its original volume. Once its volume has become stable at a given concentration a small increase in concentration will cause shrinkage, indicating an osmotic value for the protoplasm after adjustment only slightly above that of the medium. Thus we may conclude that when sea water is diluted or concentrated this amoeba automatically loses or gains osmotically active substances to or from the medium respectively such that when adjustment is completed the osmotic value of the cytoplasm is but slightly higher than that of the medium and that this is accomplished independently of the action of the vacuoles.

Phenomena of excystment in Colpoda cucullus. C. L. Claff.

Moving pictures showing induced excystment of *Colpoda cucullus* from its protective cyst by the use of laboratory grade distilled water.

JULY 19

The steady state frequency of the embryonic fish heart at different cyanide concentrations. Kenneth C. Fisher and Richard Ohnell.

When intact embryos of *Fundulus*, salmon or trout are exposed to solutions containing cyanide the heart rate is observed to become constant at a fraction of the normal frequency. There is a maximum reversible inhibition of the frequency which can be produced in this way—it can be obtained usually with N/1,000 or up to N/250 solutions. This maximum effect in the case of *Fundulus* amounts to a lowering of the frequency of the heart to about 27 per cent of the normal; in trout and salmon to about 55 per cent of the normal.

It has been observed both in *Fundulus* and in salmon that the maximum slowing produced by cyanide increases as the temperature rises. These results may be interpreted to mean that there are at least two separate series of chemical reactions involved in the determination of the frequency and that one of them contains as an integral step the Warburg-Keilin oxidase system. Cyanide, by combining with the atmungsferment, stops one of the chains and hence lowers the frequency to the value which can be maintained by the second or other chains. The effect of temperature on the maximum cyanide effect indicates that the temperature coefficient of the cyanide-sensitive chain is greater than that of the remaining series of reactions.

At constant temperature we find in *Fundulus* that as the cyanide concentration is lowered from N/1,000 to N/20,000 its effect decreases to become small or completely lacking. Referring only to that fraction of the frequency which is cyanide-sensitive, the degree of inhibition is related to the cyanide concentration by this equation

$$\frac{\text{uninhibited frequency}}{\text{inhibited frequency}} = K[\mathrm{CN}]^{-1.5} \quad (K \text{ at } 23^\circ = 1.5 \times 10^{-6}).$$

This is precisely the relation predicted by the law of mass action if cyanide is combining with and hence inactivating an essential catalyst in a series of reactions which contribute to the determination of the frequency.

These observations fit well with the conception that the frequency of the heart is determined by the velocity of two or more chains of processes which together gradually build up an unstable condition, the sudden dissipation of which, in the pacemaker, constitutes the stimulus to the heart muscle.

Studies on the refractory state resulting from the repeated injections of adrenal extract. Lena A. Lewis, Frank A. Hartman and Kenneth C. McConnell.

It has been reported from this laboratory that the injection of large amounts of adrenal extract into the normal human being and the normal dog causes marked changes in the electrolyte excretion. The effect is differential, the excretion of sodium and chloride being decreased and that of potassium and ammonia increased. The total base excreted remains essentially unchanged. It was further shown that repeated intravenous injections of adrenal extract eventually produce a condition in which there is no response.

Harrop and Thorn, however, failed to observe the development of a refractory state following subcutaneous injections.

Further studies on the conditions producing the refractory state are reported here.

Normal adult male and female dogs, adult female cats and a young immature female pig on a constant diet and regime were studied. The adrenal extracts used were Upjohn Company extract, Wilson Company extract and extracts prepared in this laboratory according to the method of Cartland and Kuizenga.

Subcutaneous injections failed to develop a refractory state, even after many injections over a long period of time (3 dogs used). These findings confirmed the observations of Harrop and Thorn.

Experiments in which a mixture of adrenal extract plus pig serum was injected subcutaneously repeatedly, gave no indication that the refractory state was due to haptene formation.

The refractory state was produced in one dog and one cat by repeated intraperitoneal injections.

Dogs which were refractory to beef adrenal extract, when injected with pig adrenal extract gave a typical electrolyte response. Similarly, dogs refractory to both pig and beef adrenal extracts gave a response to horse or sheep adrenal extract (4 dogs). The refractoriness developed to adrenal extracts is thus species specific.

A young pig failed to develop the refractory state after 8 intravenous injections of beef and 8 of pig adrenal extract. Her age may have been a determining factor in this failure to produce refractoriness.

Due to the ease with which the refractory state is produced by intravenous and intraperitoneal injections it would appear unwise to use these modes for injection of adrenal extracts in the treatment of Addison's disease.

Action potentials of visceral smooth muscles. Emil Bozler.

All-or-none conducted responses can be elicited in strips of visceral muscles by electric stimuli. It has been shown previously that conduction in these responses is muscular and occurs through syncytial connections between the smooth muscle cells. In agreement with this result the action potential during a single conducted response is a wave of negativity differing from that of a nerve only quantitatively by the time factors involved. Monophasic potentials are brief negative variations.

The spontaneous contractions of the uterus and intestine are tetanic contractions, as shown by the observation that there is a burst of impulses during each contraction. Tonic contractions are accompanied by a regular discharge. During a peristaltic wave of the ureter a repetitive discharge of high frequency was observed in the guinea-pig, a slow negative variation in the ureter of other species.

Rhythmical changes in blood flow through muscles. Laurence Irving.

Blood flow was examined in muscles and in other tissues by means of a heated electrical resistance wire type of flow meter which operates on the principle of the hot wire anemometer. In about 25 per cent of the cats under chloralose-urethane anesthesia rhythmical changes in arterial blood pressure were observed amounting to from 10 to 20 mm. in height and in a period of from 4 to 2 per minute.

As the blood pressure rose the flow through muscles diminished and the flow record indicated that the rhythmical changes in pressure originated in changes in the blood vessels of the muscles. Severing the voluntary nerves or the corresponding sympathetic supply changed the muscle flow waves in such a way that flow now passively followed pressure changes. In the brain flow likewise rhythmically and passively followed the pressure changes.

The rhythmical changes in pressure and flow were not closely related to the control of breathing; for if the breathing were altered by (1) the inhalation of CO_2 or a deficient O_2 mixture, or (2) by apnea caused by inflation of the lungs, or (3) by vagotomy the flow rhythm was not significantly changed.

The rhythmical change in flow was not closely related to arterial pressure for it persisted practically unchanged (1) when the blood pressure was depressed by the Valsalva effect or (2) when blood pressure was raised by carotid sinus stimulation from clamping the carotids. The important respiratory afferents in the vagus, depressor and carotid sinus nerves were not essential, for the rhythm appeared after their denervation.

These observations indicate that the rhythmical changes in pressure originate largely in the vascular bed of the muscles and that they are activated over the sympathetic pathway. The rhythm must originate in a vasomotor center which is relatively independent of the control of respiration. The nature of the rhythmical activity in the periphery indicates the type of rhythmical activity within the center and suggests how the activity of the center may be examined by the behavior of the peripheral system it controls.

JULY 26

Skin transplants between embryos of different breeds of fowl. B. H. Willier and Mary E. Rawles.

Small pieces (1.0 × 0.5 mm. or less) of skin ectoderm, with some adhering mesenchyme, stripped from the head of 72-hour chick embryos and transplanted to the right wing bud base of hosts of the same age but different breeds, produce large areas of donor-colored down feathers covering the entire wing and often adjacent parts of breast, back and thigh. Skin ectoderm from other regions of the embryo grafted to the head, wing, leg or tail bud gives the same result but with less spread on the head. After hatching, the down of these areas is gradually replaced by contour feathers which have the donor color and pattern but the form, rate of growth and tract arrangement characteristic of corresponding regions in host controls.

Host-graft combinations of White Leghorn, Rhode Island Red, Barred Plymouth Rock, F_1 hybrid (Barred ♀ × R. I. Red ♂), Black and Buff Minorca, White Silkie bantam, White Wyandotte and White Plymouth Rock have been tested. Areas of donor-colored feathers have been obtained in all combinations except those in which White Leghorn was donor to Barred Rock, F_1 hybrid and R. I. Red. Combinations of the various white breeds have always produced white feathers.

That the feathers of the graft area are structurally derived from host epidermis is demonstrated in two ways: (1) Structurally normal contour feathers (lack of barbicels is a Silkie character) of donor color develop when Silkie ectoderm is grafted to Black Minorca hosts. (2) Implants of limb bud mesoderm alone from Barred Plymouth Rock embryos produce in White Leghorn hosts donor-colored feather areas similar in all respects to those obtained from skin ectoderm grafts (Watterson, unpublished).

From these results and an histological study showing that the implanted skin ectoderm does not replace host ectoderm in the graft area, the interpretation is reached that the feathers of the donor-colored feather area are produced conjointly by (*a*) follicles composed of host epidermal cells and of (*b*) chromatophores or diffusible substances originating from the implant. In some way the color produced is controlled by the donor cells of the implant.

Induction by cauterization in the amphibian egg. Arthur Cohen.

Tissues and extracts of tissues of almost all groups of animals when implanted into amphibian gastrulae may produce secondary induced structures. Barth obtained inductions by implanting digitonin and suggested the latter injures the adjacent cells which then liberate the inducing substances. If this were so it should be possible to obtain inductions without implanting any substance but simply by injuring the inner layer of the presumptive non-neural ectoderm. This was done by inserting a micro-cautery needle into the blastocoele of young gastrulae of *Rana pipiens*. As a result, protuberances have appeared over the injured area.

Two criteria can be used to identify material of ectodermal origin in these embryos and early tadpoles; the presence of numerous pigment granules and the reduction or absence of yolk granules. The induced structures consist of ectodermal thickenings which give evidence of having been folded in from the surface as is the case with medullary plates; of tubes possessing well-defined lumina, possibly neural

in nature and resembling the nerve tube at the extreme tip of the tail of a normal embryo; and in one case, of striated muscle which appears to have been formed directly from the ectoderm. In all the induced structures the presence of many pigment granules and the absence of yolk granules point clearly to derivation from the ectoderm. Moreover, many of the induced structures remain either in continuity or in contact with the ectoderm. The ectodermal origin of the induced muscle is further emphasized by comparison with the normal myotome muscle of the same tadpole. In the latter case the muscle cells are still well filled with yolk but only sparsely pigmented.

Apparently the cells which are injured by the cautery needle liberate the inducing substances which then act on the surrounding ectoderm and so influence the course of its differentiation as to produce structures which do not normally arise from this portion of the ectoderm.

The innervation of transplanted limbs in chick embryos. Viktor Hamburger.

Wing and hind limb primordia of chick embryos (60–70 hours of incubation) were transplanted onto the flank or into the coelom of host embryos of the same stages. Only transplants which were located dorsally were found to be innervated. In these cases, nerves from the adjacent region of the spinal cord of the host entered the transplant and formed in it a more or less complete and typical pattern. Trunk nerves alone or in combination with limb nerves furnished the nerve supply of the transplant. It is to be concluded that the normal nerve pattern is determined by factors intrinsic in the limb, and is independent of the source of origin and of the quantity of ingrowing fibers.

Striking illustrations of the non-specificity of nerves were found in cases in which, e.g., a single branch of a brachial nerve was capable of forming an almost complete brachial pattern in a transplanted wing; or in another case in which a leg transplant replaced the host wing, and the brachial nerves of the host formed a typical leg pattern in it.

The ingrowing nerves responded to the increased demand by hyperplastic growth. This hyperplasia, however, is definitely limited; the transplants were never filled to capacity with nerve fibers. Apparently, two antagonistic mechanisms are at work: one, a growth stimulus exerted by the transplant; the other a growth-limiting factor intrinsic in the nervous system which latter sets the upper limit to the hyperplasia. The actual size of a nerve is the resultant of these two components.

The situation parallels closely that found in experiments on amphibian embryos.

The effect of mechanical stress on cartilage differentiated in vitro. Paul Weiss.

When pre-cartilage of the chick embryo is allowed to develop in tissue culture under constant mechanical stress, the resulting cartilage shows typical trajectorial patterns. The cells are oriented and arranged along lines of maximum strain. Thus, the functional adaptation of cartilage observed in the organism can be reproduced and analyzed in vitro.

AUGUST 2

Some recent studies on barnacles. J. P. Visscher.

We have worked with the larvae of some forty species of barnacle with reference to factors determining attachment. Some are attracted by certain environmental factors and others repelled. Most barnacle larvae are positive to light when first hatched, but all littoral forms with which we have worked are known to be negative to light at the time of attachment. Not only light but pressure and oxygen concen-

tration have their rôle in determining the behavior of many forms. As illustration of this fact, we have found a remarkably constant distribution of the four most common species around Woods Hole. Another series of barnacles is found only in association with crabs, and at Beaufort, North Carolina, a barnacle is found growing only in association with the Gorgonean corals.

All of these cases indicate selective attraction with reference to the cyprid larvae.

In conclusion we may note a few specific items as follows:

1. That there are six stages in the nauplius life of the barnacle—six nauplean moults.
2. That the distribution of barnacles is determined by the behavior of larvae and subsequently by the food supply.
3. That certain chemical substances are attractive and others repellent to the cyprid stage.
4. That the adults of several species of barnacles survive in fresh water but that their larvae do not.
5. That there is no evidence of distribution of barnacles from one port to another by virtue of the attachment on bottoms of ships.
6. That it is the larval life of barnacles which controls the distribution of these forms.

Observations on some of the lower Turbellaria of the eastern United States. E. Ruffin Jones, Jr.

A study of the *Acoela*, *Rhabdocoela* and *Alloeocoela* from various parts of Virginia and North Carolina as well as from Woods Hole has been undertaken. Even though the work is still in its early stages, six species hitherto unreported from this country have already been identified, including under the order *Rhabdocoela: Macrostomum stylopencillum*, a new species from North Carolina; *Omalostomum schultzei* (Claparède) 1863 from Woods Hole; *Solenopharynx flavidus* Graff 1882 from Woods Hole and Virginia; *Sopharynx oculatus* (Pereyaslawa) 1892 from Woods Hole; *Polycystis mamertina* (Graff) 1874 from Woods Hole; and under the Alloeocoela, *Enterostomula graffi* (Beauchamp) 1913 from North Carolina. In the last-named form the presence of the ductus spermaticus and vagina externa which were observed in the American representatives of the species necessitates its removal from the genus *Monoophorum* and its inclusion in the genus *Enterostomula*. The excretory system in this species consists of a pair of protonephridial tubules near the mid-ventral line and extending from the region of the cephalic ganglia to the urinary sinus, a collecting chamber which opens into the penis sheath. The tubules have a type of construction which is believed to be peculiar to this species.

Some problems of distribution and variation in the Hawaiian tree snail Achatinella. d'A. A. Welch.

Large collections of the tree snail *Achatinella* from the Island of Oahu have been collected by various people and given to the Bishop Museum in Honolulu. Dr. C. M. Cooke of that Museum has encouraged collectors to collect from highly restricted localities of not over 100–300 square yards in extent. For the past six years much time has been devoted to plotting these localities on the U. S. G. S. Top. maps of the Island of Oahu, also to collecting new material, checking as far as possible all localities and studying the organized material.

Two species have been dealt with so far. One of these, on which the work has not been completed, is in the Koolau Range and is named *A. apexfulva*. The study of the other species is now in press and the paper is entitled "The Distribution and Variation of Achatinella mustelina in the Waianae Mountains." *A. apexfulva* is used to illustrate the color variation of a species on only two ridges in the Koolau Range. On one ridge a series of six different color patterns occurs in definite areas

at successively higher elevations. The color ranges from a streaked blue gray color pattern at the lowest elevation to non-streaked pink and white patterns at higher elevation areas. The next main ridge north of this ridge, just described, has a totally different set of six color patterns, which also occur in distinct areas on the ridge at different elevations.

At present there seems to be a zonal arrangement of color patterns in the Koolau Range as well as a valley to valley variation. These color zones are present on all the ridges in the Koolau Range from which there is material and seem to occur at definite distances from the main division ridge. In the case of *A. apexfulva*, they do not appear to be correlated with any specific elevation. Streaked forms occur at the lower elevation zones while non-streaked forms occur in higher elevation zones. Although a change in color pattern in different sectors on a ridge seems to be a fairly orderly thing, the occurrence of different colors in various regions in all the lower areas of the Koolau Range as a whole appears to be a random affair. In the higher areas within two miles of the backbone there appears to be a tendency to produce white color patterns.

All this applies only to *A. apexfulva*. Another species will show an entirely different altitudinal and valley to valley variation. There are, of course, exceptions to this rule, and possibly all these ideas will be changed when the study of *A. apexfulva* is completed.

The problem of size variation with altitude was studied in *A. mustelina*. Some 15,176 shells were collected from about 582 localities. Each lot of shells from each locality was measured to the nearest millimeter and the mean length obtained for each locality. It was found that a correlation existed between shell size and the amount of annual precipitation in the locality. Shells tended to decrease in size with increase of moisture conditions. This pertains only to *A. mustelina*, for in another species the reverse is true. No size difference was noted between shells collected ten or more years ago from a definitely known restricted locality and shells collected from the same place within recent years.

AUGUST 16

Studies on fragments of centrifuged Nereis eggs. Donald Paul Costello.

When the unfertilized eggs of *Nereis limbata* are centrifuged at about 66,000 times gravity for 60 to 80 minutes, the interior protoplasm becomes stratified and the eggs are elongated into a dumb-bell form. Due to their shape, the eggs orient in the centrifuge with the polar axis perpendicular to the axis of centrifuging. To obtain egg fragments, the dumb-bells, after removal from the centrifuge, were cut by hand with microneedles. Three types of cuts were made: "upper," across the neck of the dumb-bell; "lower," across the lower edge of the yolk zone; and "intermediate," through the yolk zone. The centripetal fragment contains the germinal vesicle, and its cytoplasmic constituents differ from those of the centrifugal fragment.

Upon insemination, either fragment, or both, may be activated. While activated centripetal fragments usually cleave, the centrifugal fragments never cleave. The centripetal fragments produced by the "upper" cut rarely develop into larvae bearing prototrochal cilia although they may attain a sufficiently advanced multicellular condition. Ciliated larvae develop frequently from the "lower" centripetal fragments, less frequently from the "intermediate" centripetal fragments. These experiments indicate that some substance in the vicinity of the equator of the strongly centrifuged *Nereis* egg is essential for the development of a larva bearing prototrochal cilia. Whether this material has been displaced into this position by the centrifugal force, or whether it has retained its relationship to the polar axis despite the centrifuging, is a problem which remains to be solved.

Calcium and magnesium in relation to longevity of egg cells. Victor Schechter.

In continuation of the work of last summer on salt effects upon the longevity of unfertilized *Mactra* egg cells, high magnesium concentrations have been consistently effective in prolonging the life of the eggs and also in retaining the spherical form of the egg. The action of low calcium has not been as clear. This may be due to the fact that smaller animals of Nonamesset Flats were used currently and during the early part of the summer, whereas much larger ones coming from colder Cape Cod waters were experimented on the latter part of last summer. In view of these facts, and inasmuch as magnesium and calcium are in many fundamental ways antagonistic, the persistence of the high magnesium effect makes it possible to regard the somewhat inconsistent calcium results, not as contradictory, but as indicating a different level of initial egg condition. These results corroborate experiments performed with the *Arbacia* egg and already published. In view of the delay in breakdown of the germinal vesicle of *Mactra* in the experimental solutions, the taking up of calcium by the nucleus is postulated as an important factor in aging. Previously reported experiments on change in osmotic resistance of unfertilized *Arbacia* egg cells, with age, may also be interpreted as due to an uptake of calcium.

Some properties of living chromosomes. John B. Buck and Robert D. Boche.

A method was developed for studying salivary gland chromosomes in the unequivocally living condition. It consists in flattening the living larva between slide and coverglass and observing and measuring the nuclei and chromosomes directly through the body wall under high magnification. For quantitative work *Sciara* proved especially favorable because the nuclei of the salivary gland are spherical, uniform in size, and regularly arranged.

Study of the salivary glands of four genera of Diptera showed that in the normal living condition the chromosomes fill, or nearly fill the nucleus. Nuclear volume thus equals chromosome volume.

Asphyxiation causes the salivary chromosomes to shrink into a spherical clump, the diameter of which serves as an accurate measure of the decrease in volume. Nuclear volume as a whole remains constant, so that the exchange of fluid is entirely intranuclear. The shrinkage of the chromosomes is reversible up to a maximum of about 65 per cent loss in volume.

Injection of hypotonic and hypertonic Ringer solutions into the larva causes reversible swelling (at least 20 per cent) and shrinking (at least 35 per cent), respectively, of both nuclei and chromosomes.

Mechanical pressure on the cytoplasm causes shrinkage of the chromosomes in the affected cells.

In distinguishing between "living" and "dead" salivary chromosomes it is instructive to draw an analogy to certain features of protein chemistry. As long as the chromosomes are able to take in and give out fluid they are comparable to native proteins, but the loss of this capacity, which is associated with the death of the organism, resembles the irreversible loss of solubility attendant on protein denaturation.

Some cytological studies on virus infected cells. Alfred M. Lucas.

A study of nuclear autolysis has been undertaken in fox and guinea-pigs following infection respectively with fox encephalitis and submaxillary gland disease. The intranuclear inclusion bodies of fox encephalitis are Feulgen-positive. When they appear in the non-nucleolate cells of the anterior medullary velum, which received particular attention in this investigation, the first reaction by the normal components

of the nucleus is a pulling together of scattered chromatin granules and movement of them toward the margin. As the process proceeds the oxychromatin separates from the basichromatin and migrates in the opposite direction, namely, toward the inclusion body to which it becomes applied. Later the oxychromatin merges with the inclusion body. The basichromatin completely marginates and forms a thin shell which subsequently breaks up into small granules which aggregate into larger masses during nucleoclasis. Ultimately the nuclear membrane shrinks against the inclusion body, a reaction typical of autolysis.

Submaxillary gland virus produces intranuclear inclusions in monocytes and submaxillary gland duct cells. The inclusion bodies are Feulgen-negative in early stages but become somewhat positive later. In the monocytes the chromatin marginates but does not subsequently coalesce into clumps. The fate of the monocytes is not clear but practically all have disappeared by ten days, at which time early stages in inclusion formation may be found in duct cell nuclei. Only in the duct cells are successive steps of inclusion formation approximately synchronized. Margination process takes place during the first three or four days and this is followed by reverse movement of chromatin toward the inclusion body. The particles are small at first but later aggregate to the condition found in stock animals carrying the disease. This occurs ten days to fourteen days after the inclusions first appear. The cytoplasmic inclusions appear first when the nuclear process is about half completed.

A microdissection study of amphibian chromosomes. W. R. Duryee.

Synaptic chromosomes in the germinal vesicles of ovarian eggs of the salamander, *Triturus pyrrhogaster*, appear to be the largest yet observed. At one stage two pair are over 500 μ long in the relaxed condition. During the years of growth synaptic pairs are shown by microdissection to be fused with one another at the chiasmata.

Toward the end of ovarian development chromosomes become much shorter, consisting of transparent gelatinous cylinders in which denser less elastic segments are imbedded. Consequently in this condition vertebrate chromosomes are comparable in structure to those of the dipteran salivary gland.

However, amphibian chromosomes go through a protracted growth period (in anurans lasting over a year) during which each nodal segment gives off elastic granular side branches (av. 30 μ) in the form of loops. Since these loops are sloughed off from the chromonema cylinder, not being resorbed, both under normal and experimental conditions, we have a concrete example of material being synthesized under the direct influence of each chromomere. Such new material is added to the cytoplasm at the time of germinal vesicle breakdown, if not before.

It has been found that the relative tensile strength of the chromonemata drops from an average increase of 364 per cent in Ca-free Ringer to about 102 per cent when $CaCl_2$ 0.001 molar is present. Under the latter condition chromosomes become more brittle, and tend to come apart at the chiasmata.

AUGUST 23

The relation of the magnesium ion to ultraviolet stimulation in the Nereis egg. Karl Wilbur.

Earlier experiments have shown that the germinal vesicle of the *Nereis* egg, which normally remains intact when the egg is shed into sea water, breaks down when treated with a variety of stimulating agents such as ultra-violet radiation and isotonic solutions of NaCl or KCl. The breakdown is apparently dependent upon calcium, for if this ion is first removed from the egg cortex with sodium citrate these stimulating agents are ineffective.

The magnesium ion inhibits germinal vesicle breakdown produced by ultraviolet stimulation. This was demonstrated by immersing eggs in mixtures of sea

water and isotonic $MgCl_2$ and exposing them to the unfiltered radiation from a mercury arc lamp. The relation of the concentration of magnesium to effectiveness of stimulation was examined quantitatively.

The inhibitory action of magnesium may be overcome by increasing the irradiation time. Prolonged exposures in sea water give a lower percentage breakdown than more moderate irradiation. Nuclear breakdown with ultra-violet stimulation is not immediate and can be completely inhibited by the addition of isotonic $MgCl_2$ mixtures 3 to 5 seconds following irradiation in sea water.

The inhibitory effect of a sea water-$MgCl_2$ mixture is reduced by the addition of isotonic $CaCl_2$. Enough $CaCl_2$ was added so that the Mg-Ca ratio was made to equal the ratio of these ions in sea water.

Eggs in magnesium-free sea water show a greater percentage of germinal vesicle breakdown with ultra-violet stimulation than eggs in sea water containing magnesium. A few of the non-irradiated eggs in magnesium-free sea water at a pH slightly below 6.0 may undergo spontaneous breakdown, and in a more alkaline solution many of the eggs show spontaneous breakdown, polar body formation and an irregular type of division.

The results indicate that the response of the *Nereis* egg to stimulation is similar to other types of protoplasmic response in its relation to the magnesium ion.

Cytological effects of x-rays on grasshopper embryos. E. Eleanor Carothers.

A study of *Melanoplus differentialis* embryos which had received a single dose of x-rays in early pre-diapause leads to the following observations and conclusions concerning the immediate and latent effects of radiation. The first effect is to speed all cells which are in late prophase or metaphase at the time of x-raying through to anaphase. An apparent inhibition follows since cells which would divide normally during the time of the lag have been rushed through. All cells in late prophase, metaphase or anaphase at the time of radiation die at anaphase with pycnotic nuclei. It is thus purely a matter of chance which cells will be eliminated by this means; except that the cells of regions where certain anlaga are being formed are more active mitotically at particular times with consequently greater injury to those regions, or that cells which are fully differentiated functionally and have ceased to divide (serosa and yolk cells) are protected. Many of the cells with pycnotic nuclei are engulfed by adjacent tissue cells. Two days after radiation metaphases show fragmented and lagging chromosomes but there is no new pycnosis. A second type of injury, characterized by liquefaction of the cytoplasm, is now evident. Many, if not all, of the cytolyzing cells contain ingested pycnotic nuclei. Within five days extensive cytolytic lesions appear. This cytolysis may be due to direct injury of the cytoplasm by the x-rays or to a toxic effect from the phagocytized necrotic cells. Mitosis seems to be stimulated, perhaps due to the liberation of intercellular hormones. Loofbourow ('37) and others have shown that injured cells may yield substances which cause rapid proliferation of uninjured cells. Yolk cells phagocytize injured cells and remove them from the vicinity of the embryo. Growth ensues in many of the embryos with most of the cells now apparently normal. Shortly before diapause and in early post-diapause the nuclei of groups of cells become pycnotic, due probably to lack of necessary cellular associations. Finally, the embryos die, due to lack of organization rather than to absence of differentiation, since all types of tissue may be present.

Quantitative studies on the effect of x-rays on mammalian cells, and on the mode of x-ray action. J. Furth.

The effect of x-rays on malignant blood cells in mice can be studied quantitatively. The number of cells can be estimated (*a*) by injecting a counted cell suspension in

diminishing amounts into closely related inbred mice and (*b*) by determining the length of life of mice after inoculation, this being inversely related to the number of living cells injected. Malignant lymphocytes are highly susceptible to x-rays: 200r kills all but about 1 of 100 and 400r, 1 of 1,000 x-rayed cells. Approximately 800r is fatal to all cells. The malignant myelocytes studied are slightly less susceptible to x-rays than the lymphocytes and the monocytes are very resistant. The few cells that escaped destruction behaved like unirradiated cells. The lethal dose of x-ray is approximately the same *in vitro* for leukocytes concentrated in Tyrode solution as *in vivo* for the same number of leukocytes scattered throughout the body. This observation suggests that x-rays affect cells directly. Experiments with parabiotic twins made in association with Drs. A. A. Brower and O. B. Furth, in which one animal was irradiated and the other shielded, support this opinion. Tissue culture studies indicate that death produced by x-ray is delayed by several days or weeks during which the cells migrate actively and probably multiply.

The effect of x-rays on Arbacia punctulata sperm. Paul S. Henshaw.

Delay in the onset of the first cleavage in *Arbacia* eggs may be brought about by subjecting either or both of the gametes to x-rays. One distinct difference occurs, however, depending on whether the sperm or eggs are irradiated. In the case of the eggs recovery has been found to take place both during treatment and during the interval between the end of treatment and the moment of insemination. In the case of the sperm, on the other hand, no recovery takes place during either interval. Thus a much clearer picture of how the delay is produced as a function of the dose of radiation may be obtained by treating the sperm. Ten experiments have been accumulated which are consistent in indicating that the amount of delay produced is directly proportional to the logarithm of the amount of radiation applied.

Further, it has been found that when the dose of radiation is sufficiently large, multipolar cleavage is produced in a large percentage of the eggs. This happens irrespective of whether sperm or eggs are treated but to a larger extent usually when sperm are exposed. Examination of serial sections of the treated material showed that the multipolar condition was not due to polyspermy and that the first manifestation of it was the formation of accessory asters which was followed by multiple spindles and the multipolar cleavage condition. Since these changes may be produced equally often or even oftener when sperm are treated, and since sperm are composed mainly of nuclear material, the evidence leads to the presumption that the multiplicity effects are brought about by changes produced in the nucleus.

Whether delay in cell division and multipolar cleavage are both due to the same initial change produced by the radiation is not clear. Yet, if the inference just mentioned should apply—namely, that multipolar cleavage is due to nuclear changes produced by the radiation—the two reactions would have this in common, for it has been shown in previous work with nucleated and non-nucleated egg fragments that the delay in cleavage results from nuclear changes.

Recovery of Arbacia eggs from high intensity x-ray effects. T. N. White.

Recovery following x-ray treatment at about 500 roentgen/min. has been discussed by P. S. Henshaw (*Amer. Jour. Roent. and Radium Therapy*, Vol. XXVII, p. 890, 1932). Results of similar experiments with x-ray output of about 7,600 roentgen/min. were shown in comparison, and the principal features of recovery under the new conditions were pointed out.

General Scientific Meeting

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Isolation of salivary gland nuclei. A. M. Melland.

Salivary glands of dipteran larvae, chiefly species of *Chironomus*, with nuclei up to 100μ in diameter, were used. As the chromosomes practically fill the nucleus, whole nuclei may be collected for analysis of chromosome material.

Glands were dissected out either in isotonic Ringer's or in the body fluid of the larva; treated as follows; and the nuclei dissected out with fine steel needles.

Unfixed Glands.—The most satisfactory method for obtaining nuclei which, when dissected, will react osmotically to various salt solutions is that of dissecting the glands on an ice-cooled stage after being in .25 per cent dried egg albumen solution for 2–3 hours at 5° C. The nuclei then come out easily and are not too sticky.

Fixed Glands.—In the dissection of impregnated and tanned material the criteria used were the ease of dissection and the resemblance of the dissected to the living nuclei. Glands in isotonic Ringer's were left over the osmium vapour from a 2 per cent solution for 24 hours at 5° C. and the nuclei then dissected out in distilled water. These nuclei do not show any shrinkage of the chromosomes. Glands in 10 per cent formalin for 24 hours at 14° or for 36–60 hours at 8° yielded nuclei which were dissectible in 10 per cent formalin: 50–80 nuclei being dissected per hour.

Cytological studies on the living thyroid of the salamander. Glenn H. Algire.[1]

A method is described for the transillumination of the thyroid gland of the living salamander, with the normal blood supply intact, making it possible to observe and take photographs with the highest oil immersion systems of the compound microscope. The illuminating system differs from that of the conventional microscope in having the two top condenser lenses replaced by a straight quartz rod, through which rays of light are transmitted to its tapered tip by internal reflection. The rod projects through a hole in the animal dish and is sealed against leaks. The operation isolates the thyroid and its vessels from fascial connection with neighboring structures. The gland is brought directly over the tip of the rod by means of Harne micromanipulators. Artificial respiration is accomplished by passing in air through a cannula inserted into the tip of the lung. Specially constructed immersion caps permit the use of the oil immersion objectives for observations of the gland which is immersed in Ringer's solution. The apparatus makes it possible to study, in the living intact animal, the cytological changes which occur in the thyroid cells in response to injections of thyreoactivator. In this manner it has been confirmed that the structures observed in fixed and stained sections are not artifacts, but exist in the living cell. These structures, as described by Uhlenhuth, are: the plasma granule, the colloid droplet, intracellular vacuoles, and intercellular spaces.

Micro-structural changes in muscle fibers after caffeine. Ralph H. Cheney.

The gastrocnemius muscle of *Rana pipiens* Schreber which has been used by the author for a series (1931–37) of physiological studies on caffeine and nicotine effects, was immersed in caffeine-in-Ringer. The concentrations and time intervals employed were represented by 0.03 per cent for 10 minutes and 30 minutes respectively; 0.06 per cent for 30 minutes; 0.12 per cent for 10 seconds; 0.50 per cent for 2 seconds; and 1 per cent for 30 minutes. The preparations were fixed in Bouin's and stained with Heidenhain's iron haematoxylin.

[1] From the Department of Anatomy, University of Maryland Medical School.

Loss of myofibrillar tension results in a "wavy" appearance, indicating a folding of the Q and J band material without loss of alignment of the cross striations. This phenomenon occurs in normal muscle when excised. Depending upon the concentration and time of immersion, the myofibrillae pass through a definite order of microphysical changes. The chronological stages may be described histologically as (1) *incomplete* or *partial clot* formation, in which the regular cross striation pattern is replaced by local irregular zones in which marked shortening of myofibrillar substance has occurred; and (2) *retraction cap* appearance, involving the rupture of the majority or all interfibrillar Z membranes and the redistribution and "congealing" of Q and J band substances with the formation of Zenker's Waxy Degeneration areas. Retraction caps are abundant in muscles in the state of caffeine contracture.

The sequences of microstructural changes induced by caffeine are similar to (or identical with) the action shown in fixed preparations of striated muscle by Nageotte ('37) to occur in response to extreme faradic current and to chloroform. The stages of alterations in the myofibrillae after caffeine immersion correspond also to the observations of Speidel ('38) on the living muscle fiber after bruising, cutting, electrical stimulation, alcohol, and non-isotonic salt and sugar solutions.

The suggestion presents itself that the sequence of ultimate physical changes in the shortening process of the myofibrillae is a constant factor and largely independent of the nature of the preceding chemical reactions in the sarcoplasm.

Some features of contraction nodes and retraction clots as observed in single fibers of cardiac and skeletal muscle of both vertebrates and invertebrates. Carl C. Speidel.

An abnormal variety of muscle fiber contraction is featured by the formation of a sharply localized node of contraction which comprises relatively few (10–20) sarcomeres. Such a node may form and travel along a fiber to the end, or it may form and disappear without traveling. Successive nodes may travel along a fiber in the same direction, several being present at one time. Two nodes may travel toward one another in the same fiber, collide and disappear. A node may form and divide, each of the two halves then traveling along the fiber in opposite directions. A node may, or may not, succeed in passing a retraction clot in the fiber.

The phenomenon of muscle clotting after injury is characterized by the transformation of cross-striated material into waxy zones of coagulation (Zenker's degeneration). The most conspicuous variety of retraction clot is accompanied by rupture of myofibrils on one or both sides of the clot. A less conspicuous incipient retraction clot may form, however, without rupture of myofibrils. This is a soft thin clot waxy in appearance at first. It may be quickly replaced (within a few minutes or even a few seconds), however, by a cloudy zone. This zone is without cross striae but is still capable of further shortening and clotting. Between these two extremes many intermediate retraction clots occur. Retraction clots vary greatly in number of myofibrils involved. They may be thick or thin, and may be placed across the fiber transversely, irregularly, or in pyramidal, zig-zag, or terraced fashion. Intercalated discs in the heart probably arise as thin retraction clots.

This study is based chiefly on arthropods (*Limulus* and *Palaemonetes*), amphibians (*Rana, Hyla* and *Pseudacris*), and mammals (*Mus*).

Illustrative cinéphotomicrographs have been obtained (see abstract of motion picture, p. 359).

The heteropycnosis of sex chromosomes and its interpretation in terms of spiral structure. Michael J. D. White.

There are two kinds of heteropycnosis which may be called negative and positive heteropycnosis. In the X-chromosome of the Acrididae both types are met with, the chromosome reversing its behavior during the course of spermatogenesis. Both

positively and negatively heteropycnotic chromosomes have a spiral structure at metaphase. The number of gyres in the spiral per unit length is inversely proportional to the thickness of the chromosome, so that it is greater in negatively heteropycnotic chromosomes than in positively heteropycnotic ones. Probably all metaphase chromosomes are compact spirals in which the gyres are in contact with one another, so that there is no space between them and only a "virtual" space in the axis of the coil. The spiral structure is not apparent, however, until the coils have been relaxed somewhat, so that there is a space between them. The direction of coiling (right- or left-handed) is apparently at random, since in the X-chromosomes of long-horned grasshoppers both kinds are met with in approximately equal numbers in the same individual.

Mitochondria and other inclusions in the ciliate Tillina canalifera. John P. Turner.

Studies on the living protoplasm and of 4μ sections of *T. canalifera* made according to the methods of Champey-Kull, Benda, Regaud, Mann-Kopsch, Ludford, Kolatchev, Hirschler, etc., and *intra vitam* staining have revealed:

(1) Endoplasmic granules $\frac{1}{2}$ to $\frac{3}{4}$ μ in diameter which respond to the mitochondrial techniques, except that they do not stain clearly with Janus green. Many of these granules are elongated into rods and dumb-bells $1\frac{1}{2}$ μ long. Champey-Kull and Benda preparations show a faint clear area around each granule as though it were contained in a vacuole.

(2) Similar granules in the cortex which are more irregular in shape and which take a delayed neutral red stain.

(3) Rods up to 4 μ long and $\frac{1}{2}$ μ thick visible in the living endoplasm as conspicuous, highly refractive bodies. These have an osmiophilic cortex and an osmiophobic center.

(4) Minute osmiophilic granules in the endoplasm which are usually seen collected around an osmiophobic globule so as to form a cortex about it. Forms intermediate between numbers 3 and 4 are also found.

(5) Neutral red-stainable granules in the endoplasm which are extremely minute at first, but which appear to grow in size as the preparation ages. These are frequently concentrated around the cytopharynx and the forming food vacuole.

(6) Fat droplets of irregular size in the endoplasm which stain brilliantly with Sudan III.

Cytoplasmic inclusions and matrix of the Arbacia egg. Robert Chambers.

A striking example of the property of protoplasm to undergo sudden and reversible sol-gel transformation occurs in the light fragments produced by centrifuging sea-urchin eggs. The heavy fragment, containing the bulk of the cytoplasmic inclusions, can be readily churned by pushing with a blunt microneedle. On the other hand, the light fragments, although highly liquid, tend to resist being churned by becoming immediately converted, upon manipulation, into plastic solids. Slight compression causes such distorted, solid bodies to revert to liquid spheres.

The cell inclusions to which particular attention has been given are (1) the fat globules of the so-called oil-cap of centrifuged eggs and (2) the pigment vacuoles or so-called pigment granules.

The fat globules are almost at the limit of visibility, being considerably smaller than one micron in diameter. Each fat globule is enveloped by material which becomes coagulated into a single mass on exposure to heat (70° C.) or to formalin. Gently compressing the brittle mass with a needle causes the included oil to exude and to coalesce into a relatively large drop of oil. The oil-cap is agglutinated by $CaCl_2$ and dispersed by NaCl or KCl.

The pigment vacuoles are about two micra or less in diameter. They are globular and easily deformable. On puncturing with the tip of a fine microneedle, the enveloping membrane disappears, releasing the pigment which diffuses into the cytoplasmic matrix. The same phenomenon occurs when the eggs are exposed to heat and during cytolysis induced by mechanical injury of the eggs. Fat non-solvent, coagulating agents maintain them intact. It is noteworthy that the pigment vacuoles, like many plant vacuoles, are more acid than the surrounding cytoplasm and resemble the plant vacuole in their avidity for neutral red.

The Devaux effect at oil-protoplasm interfaces. M. J. Kopac.

Devaux [*C. R. Acad. Sci.*, **202**: 1957, 1936] reported that minute folds or wrinkles are produced by a monolayer of protein covering an oil-water interface. Such wrinkling is the result of a spontaneous increase in interfacial area and indicates zero interfacial tension. Langmuir and Waugh [*Jour. Gen. Physiol.*, **21**: 745, 1938] named this phenomenon the Devaux effect. Oil-drops in the presence of excess protein also may spontaneously increase their surface area as shown by wrinkling. With low protein concentrations, the interfacial tension, although considerably reduced, remains positive.

The results of testing the Devaux effect on cellular systems are as follows: Oil-drops in contact with protoplasm show no Devaux effect, but a pronounced wrinkling quickly appears after the cell is cytolyzed. Oil-drops in small, incipient cytolytic zones become slightly wrinkled, the maximum effect occurring after extensive cytolysis. Striking results occur when the oil is introduced just prior to cytolysis, the change to zero interfacial tension requiring less than 15 seconds.

There is a significant difference in the effect produced on oil-drops introduced before or after cytolysis. Moreover, the time at which an oil-drop is introduced into a cell is also an important factor. Oil-drops in contact with protoplasm for 5 minutes will not wrinkle after cytolysis, while drops brought in contact from one-half minute before to one-quarter minute after cytolysis quickly indicate a zero interfacial tension. Oil-drops injected from ¼ to 1¼ minutes later show only a slight wrinkling.

The study of the Devaux effect at oil-protoplasm interfaces suggests two aspects of protoplasmic structure. The first and more obvious one is that proteins do not accumulate on experimentally introduced oil interfaces while the protoplasm is intact. Apparently the proteins are bound in the living cell and released only on cytolysis. The second and less obvious conclusion is that the protoplasmic proteins are essentially unstable, high molecular weight complexes. This point of view makes it possible to show how the Devaux effect is obtained at the instant of cytolysis. The adsorption of a complex aggregate which later dissociates produces the same effect as a high initial concentration of low molecular weight protein. On the other hand, if the large molecules dissociate before they are adsorbed, the initial concentration is, in effect, low. The adsorption of proteins on oil-drops introduced after cytolysis has been demonstrated [Kopac, this number].

Further studies on the permeability of the erythrocyte to ammonium salts. M. H. Jacobs and A. K. Parpart.

By means of the photoelectric method of Parpart (*Jour. Cell. Comp. Physiol.*, **7**, 153, 1935), it has been shown that volume changes of beef erythrocytes caused by the penetration of $(NH_4)_2H$ Citrate are almost negligibly slow. Advantage has been taken of this fact to repeat on the erythrocyte experiments of the sort reported by Jacobs and Stewart (*Jour. Cell. Comp. Physiol.*, **7**, 351, 1936) for the *Arbacia* egg in which an unequal distribution of a penetrating ammonium salt between a cell and its surroundings was brought about by the presence in the external solution of a non-penetrating ammonium salt. The addition of a small amount of concentrated ammonium acetate, valerate, salicylate, etc., causes swelling of erythrocytes in originally

isotonic solutions of $(NH_4)_3H$ Citrate but not of NaCl. For reasons discussed in the earlier paper, ammonium borate shows a different behavior. Because of the permeability of the erythrocyte to the Cl ion, the addition of a small amount of saturated NaCl also causes swelling of cells suspended in isotonic ammonium citrate solutions, even though the osmotic pressure of the surrounding medium is thereby increased. This effect is greater when the solution is first made less acid by the addition of NH_4OH. When a small amount of NH_4 salicylate is added to erythrocytes in M/6 NH_4Cl the time of hemolysis may either be decreased or greatly increased according to the time of addition. The volume changes of the cells under these conditions suggest that the NH_4 salicylate has a swelling effect of its own similar to that mentioned above but that at the same time it greatly decreases the permeability of the cell to the Cl ion. No corresponding decrease in permeability to glycerol or thiourea or to ammonium salts which enter the cell by a non-ionic mechanism has been found. Ammonium benzoate has a very similar effect with the erythrocytes of many mammals, but only a slight permeability-decreasing one with those of the rat.

Permeability and the lipoid content of the erythrocyte. A. J. Dziemian and A. K. Parpart.

Stroma were obtained quantitatively from the erythrocytes of the sting ray, smooth dogfish, and rabbit by repeated washings with distilled water and hypertonic sodium chloride. The lipids were extracted from samples of intact erythrocytes of the above species by both the refluxing and cold alcohol-ether methods. The same was done for stroma from equivalent amounts of cells. Aliquots of the above were extracted with petroleum ether and the total lipid content was determined by the gasometric technique. The cold cell, refluxed stroma, and cold stroma extracts showed identical amounts of lipid, but the refluxed cell extract, in most cases, had a greater quantity, possibly due to breakdown products of hemoglobin. It appears that the cold extraction gives a truer picture of the erythrocyte lipids than does the reflux extraction.

Cold extracts were made from beef, rat, rabbit, cat, opossum, smooth dogfish, and mackerel erythrocytes. Fish cells had two to three times more lipid per unit area than did mammalian cells. The mammalian red cells of the species examined had almost identical amounts of lipid per unit area, except in the case of the beef cells, which contained a slightly higher quantity.

While the results, considering the rat and beef erythrocytes, appear to agree with Höber's theories on the penetration of lipid-soluble substances, the difference in lipid content between the two species does not seem to be great enough to account for differences in permeability.

The relation of the shell to anaerobic metabolism in Venus mercenaria. Louis-Paul Dugal and Laurence Irving.

During survival of *Venus mercenaria* when kept out of water the CO_2 content of the mantle cavity fluid increases without much decrease in pH. The shell is visibly eroded under the center of the mantle. The calcium of the fluid increases more than the CO_2, indicating the formation of other acids. The small change in pH indicates that the other acid is neutralized and buffered, and glass electrode titration shows that its pK corresponds with that of lactic acid. The volatile weak acid in the fluid as determined by titration agrees with the CO_2 determined by the Van Slyke method. The sum of the CO_2 and other acid is equivalent to the calcium. From these relations it is evident that the CO_2 gained by the fluid is derived from the shell and is not produced by anaerobic or aerobic metabolism. While the clam is out of water a process resembling glycolysis in vertebrate tissue forms an acid. The acid is neutralized by the shell through the intervention of the living mantle. Part of the CO_2 dissolved from the $CaCO_3$ of the shell remains in solution. Gains in CO_2 and in non-volatile weak acid

are equivalent to gain in calcium, so that these are the only participants in buffering. The whole process depicts the well-regulated use of calcareous shell for buffering the products of glycolysis during a period of anaerobic metabolism.

Tension output of muscles in hypotonic solutions. Alexander Sandow and Kenneth Moritz.

These experiments extend the research of the following on the activity of frog muscle in relation to varying tonicity: Cooke, *J. Physiol.*, **23**: 137, 1898; Meek, *Am. J. Physiol.*, **17**: 8, 1906; Chao, *Chin. J. Physiol.*, **10**: 547, 1936. The effect of absorption of water from hypotonic solutions on the output of *Rana pipiens'* gastrocnemii was studied by a method analogous to that used by Saslow and Webster (*J. Pharmac. and Expt. Therap.*, **53**: 142, 1935) in their study of caffeinized muscles. The two gastrocnemii were quickly removed, weighed, and immersed in Ringer's solution for an hour. Kymograph records were then obtained of the tension developed in response to 10 maximal break induction shocks (1 per second). Only those mates whose difference in output (tension per gram) during this initial period was less than 10 per cent were retained for further testing. One muscle of a symmetrical pair was then placed in a hypotonic solution made by diluting Ringer with distilled water; the mate used as a control was placed in an isotonic mixture containing the same fractional part of Ringer as the hypotonic solution, but the remainder made up of isotonic sucrose. After soaking about 3 hours, the muscles were weighed again and then stimulated with maximal shocks as before until complete fatigue. The records were measured for maximal response tension and total tension output. Most of the experiments involved tonicities 0.9 and 0.8 that of standard Ringer. Control experiments showed that muscles in isotonic solutions containing up to 20 per cent isotonic sucrose and the remainder Ringer, developed the same output as their symmetrical mates in pure Ringer.

The results demonstrate that the maximal response (in general agreement with Chao) first increased with increased water uptake and then decreased. The greatest maximal response occurred at a water absorption of about 10 per cent of the muscles' initial weight and amounted to 130 per cent of the maximal response of the control. The variation of the total output followed the same general curve as the above, but the greatest output, also at 10 per cent weight increase, was 145 per cent of the control. Further experiments are planned to elucidate the mechanism of these phenomena.

The mechanism for the control of the intake and the output of water by the vacuoles in the marine amoeba, Flabellula mira Schaeffer. Dwight L. Hopkins.

The marine amoeba *Flabellula mira* Schaeffer can live equally well in sea water diluted 20 times with distilled water or in sea water concentrated several times. Water is eliminated from the cell by expulsion of food vacuoles which on being expelled are largely filled with fluid. The intake of water which causes the swelling of the vacuoles in actively feeding amoebae is due to the production and isolation into the vacuoles of a by-product of metabolism. This substance is isolated into the vacuoles, giving them an osmotic value at the time of their origin many times that of the surrounding protoplasm and medium. They consequently absorb water according to the laws of osmosis. When the amoebae are not feeding due to unfavorable conditions, this substance is isolated into the vacuoles in the form of visible granules, *condensation granules*, in which case it exerts no osmotic effect and the vacuoles remain very small and stationary as to size.

This substance, when precipitated in the form of granules, shows properties generally ascribed to mitochondria, staining with Janus green B and by other techniques for staining mitochondria. The granules are soluble in ether, chloroform and alcohol, but not in acetone. They are destroyed by reducing agents and preserved by oxidizing agents.

In actively feeding amoebae the rate of water intake and elimination by the vacuoles is inversely proportional to the concentration of the outside medium and directly proportional to the volume of the cell, thus indicating that the osmotically active substance in the vacuoles is produced in a constant quantity in a unit of time by a unit volume of protoplasm. In non-feeding individuals the water intake and elimination by the vacuoles are zero or nearly zero and do not vary with the outside concentration.

On the kidney of the crayfish and on the uptake of chlorid from fresh water by this animal. N. S. R. Maloeuf.

A thorough study is presented on the anatomy of the kidney of the American crayfish (*Cambarus bartoni*) and on the arterial supply to that organ. This study is made as an anatomical prelude to physiological investigations similar (it is hoped) to the celebrated ones made by Prof. Alfred N. Richards and his school on the amphibian kidney. The nephridial canal (which is about 3 cm. long and a little over 1.5 mm. at its greatest diameter) occupies by far the largest mass of the kidney. It is here shown for the first time that the cells of its proximal (ventral) half are markedly different from those of its distal (dorsal) half. Hans Peters (1935) was the first to note that the nephridial canal is composed of two types of epithelium but made no statement as to which parts of the nephridial canal these types correspond. The cells of the proximal half form flat pavement epithelium and exhibit no apparent secretory activity while those of the distal half are large, columnar, and display large clear secretory vacuoles at their apices and a marked mitochondrial structure. This fact and the fact that the crayfish passively takes in fresh water through its gills (Maloeuf, 1937) makes it probable that the cells of the distal half absorb water from the blood and reject such into the lumen of the nephridial canal. This alone might account for the markedly hypotonic urine, i.e. a reabsorption of salts by the nephridial canal does not seem necessary. This appears especially probable since these animals are capable of absorbing Cl from fresh water by way of their skin (presumably by way of their gills). The epithelium throughout the kidney is monolayered.

When in fresh water, crayfish do not take in water by way of their mouths. The animals (*C. bartoni*) shipped from Wisconsin to Woods Hole in dry boxes (and which had thus remained about a week without water) were placed in running aerated tap water without food for about 10 days. At the lapse of this time and following they were found to exhibit a net excess of Cl absorption from tap water (a microburette run by a reversible telecron motor was used); i.e., they were absorbing more Cl than they were losing by way of their kidneys. The net excess was at first about 0.35 mg./20 gm./24 hours. The net excess fell with time and, in 20 days after the first analysis, it was about 0.048 mg./20 gm./24 hours. When their fast (of at least a month) was broken by a meal of minced raw beef the rate of excess Cl absorption declined slightly but significantly. These facts indicate that the net excess of Cl absorption is a compensation for the loss of Cl which had presumably occurred by way of the kidneys when the animals were in the dry boxes and thus unable to make up for the loss by an absorption of Cl through the skin. This discovery, while merely due to matters of circumstance, appears to be capable of offering important clues as to whether filtration is of importance in the renal function of the crayfish. When kept out of water, how long will the crayfish kidney secrete urine at a constant rate and how does this rate vary with the hydrostatic pressure of the blood? Except for the bladder and the proximal half of the nephridial canal all portions of the crayfish kidney exhibit secretory activity and nowhere is there anything resembling the tenuous epithelium of Bowman's capsule of the vertebrate kidney. With the discovery of a marked carbonic anhydrase activity in the gills of invertebrates (Ferguson), Cl absorption may also be of importance in supplying the Cl shift for the escape of CO_2.

The osmo-regulative function of the alimentary tract of the earthworm, and on the uptake of chlorid from fresh water by this animal. N. S. R. Maloeuf.

When an earthworm (*Lumbricus terrestris*) has been in tap water for some time (about 12 hours) and is weighed at successive relatively short periods thereafter it invariably loses weight until a certain minimum (about 15 per cent of the initial) is attained. The initial weight is completely regained or even exceeded if the worm is left undisturbed for several hours. These observations of Adolph (1927) have been confirmed. He suggested that the water is absorbed through the skin, is collected in the gut, and is expelled by way of the anus. By ligating the anterior and posterior ends of worms which had been in tap water for a protracted period and of worms directly before their introduction into tap water and by studying the weight changes it is possible to show that water which passes into the gut is expelled by both the mouth and the anus with approximately equal ease. As shown by the swollen condition at the anterior end and by punctures into the gut, the excess water is evidently partly stored in the crop. Further proof that water can be expelled from the mouth is shown by the sudden decrease in the net weight of the worms upon removal of the anterior ligature. A jet of clear colorless liquid was not infrequently noted to issue from the mouths of worms which had been in tap water upon ligating the anterior end. The percentage weight increase is greatest in worms which were ligated at both ends directly before their introduction into tap water from the soil. The weight then generally attains an approximately constant value (with minor fluctuations which can be due only to loss of liquid by way of the kidneys, the loss of which could be clearly observed under a binocular and which occurred from no other surface of the body) of approximately 115 per cent of the initial weight indicating that water is no longer absorbed. Dying worms showed no such constancy but continued to increase in weight. Amputating the nerve cord in the anterior end acts in the same manner as an anterior ligature.

The percentage of water in the gut contents of worms which had been in tap water for several hours is markedly greater than that in the soil from which the worms were removed. The percentage is still greater in worms in tap water and ligated at the posterior end; and is greatest in worms ligated at both ends. Correlated with this, the total quantity of materials (mainly water) in the gut of worms in tap water was much greater in worms ligated at both ends than in worms which were not ligated and still greater than in worms in the soil.

Worms, ligated at both ends to eliminate possible interference from the gut, will decrease in weight in even hypotonic sodium chlorid, urea, or glucose (added to tap water). The decrease is not necessarily proportional to the concentration of any one of these compounds. It is clear that no simple mechanism is involved. Per equimolar concentration, the decrease in urea is markedly less, this presumably being due to the greater penetrability of urea. With similar treatment the worms increase in weight in tap water.

Earthworms which had been in tap water for about 12 hours and which were then ligated at both ends showed a net excess absorption of chlorid from tap water. When ligated at both ends directly before introduction into tap water from the soil, all the worms showed, on the contrary, a loss of chlorid. This, together with the facts that worms so treated were especially viable and that untreated, apparently normal worms frequently died within 12 hours after introduction into running aerated tap water while all controls in moderately damp soil remained vigorous, indicates that the transition from soil to fresh water is not an easy one for the earthworm.

Earthworms introduced into tap water without any ligatures show but a slight net excess absorption of chlorid. This together with the fact that the unhealthy ones lost chlorid is nice evidence for the active retention of chlorid by the skin.

The kidneys probably play only a minor rôle in the osmo-regulation of the earthworm.

Development of half-eggs of Chaetopterus obtained by centrifugal force. Ethel Browne Harvey.

The *Chaetopterus* egg can be broken into two spheres of constant size by centrifugal force if suspended in a sugar solution. The white sphere contains oil, clear layer, nucleus or polar spindle, mitochondria (granules staining purple with methyl green) and a few yolk granules; the yellow half contains only yolk granules. The measurements are

	Diameter μ	Volume μ^3
Whole egg	94	434,900
White half	83	299,400
Yellow half	64	137,300

Both the white and yellow halves can be fertilized, and also can be activated artificially with KCl. The white half, both fertilized and parthenogenetic, forms polar bodies and cleaves according to the peculiar and typical annelid pattern. At first cleavage, it divides into two unequal cells, and at second cleavage, the smaller cell divides equally and the large cell unequally into a large and an intermediate-sized cell. It tends to become, especially the parthenogenetic white half, amoeboid, and often has large nuclei and irregular cells. Both the fertilized and parthenogenetic white half develop into swimming blastulae.

The yellow half, both fertilized (fertilized merogone) and parthenogenetic (parthenogenetic merogone), usually cleaves only once into two equal or unequal cells. It becomes amoeboid and develops no further.

The question of whether the delay in cleavage of Arbacia eggs produced with X-rays is caused by a general slowing of the cleavage process or by a block at some particular stage. Paul S. Henshaw.

Arbacia punctulata sperm have been exposed to doses of x-rays sufficiently large to cause a marked delay in the onset of the first cleavage (when allowed to fertilize normal eggs). Samples of eggs so fertilized together with controls (i.e., normal eggs fertilized with normal sperm) were fixed, sectioned and examined to determine at what stage during the cleavage cycle the slowing occurred. Similar studies were made in which the radiation was applied to the eggs rather than to the sperm.

Examination of a large number of organisms from a wide variety of samples disclosed the following: that about the same effects were obtained irrespective of whether sperm or eggs were treated; (2) that the entrance of the sperm and the fusion of the pronuclei take place quite as rapidly in case of the irradiated material as in case of the non-irradiated; (3) that the entire remainder of the cycle is slowed, but considerably more during the phase between the fusion of the pronuclei and the appearance of chromosomes; and (4) that if this phase of greatest delay be divided into two parts—the first from fusion of the pronuclei to the appearance of asters which attend mitosis and the second from the appearance of these asters to the appearance of chromosomes—definitely more delay is produced in the second part. The period of greatest delay is therefore associated with the prophase of mitosis and the manifestation of the greatest irradiation effect so far as delay in cleavage is concerned occurs when the nuclear material is undergoing changes in preparation for the formation of chromosomes.

The resistance of fertilized Arbacia eggs to immersion in KCl and NaCl solutions. E. L. Chambers and Robert Chambers.

The experiments performed consisted in immersing fertilized *Arbacia* eggs in isosmotic solutions of NaCl and KCl. The procedure differs from those of previous

investigators in that the eggs were passed through many washes of the given salt to eliminate all traces of sea water and were left continuously in the salt solution. Temperature and pH were carefully controlled.

Washed eggs were immersed in the solutions at varying intervals after insemination. The effect was noted by the percentage of cleaving eggs. The toxicity of the NaCl-solution was very great and was directly proportional to the length of sojourn in the solution. Division occurred only among those eggs immersed shortly before or during the first cleavage period. Eggs immersed in KCl-solution exhibited a reverse effect. Those immersed shortly after insemination were found to be highly resistant and the percentage of cleavage fell rapidly to zero when immersed at any time later than 10 to 20 minutes after insemination.

The resistant period to K-ions has several characteristics: (1) it varies greatly in batches of eggs from different sea-urchins, (2) it occurs only at one stage in the cycle of development of the fertilized egg, there being no resistant period between the first and second cleavage, (3) it is not related to the cycle of mitosis, nor to the time of union of the pronuclei and (4) its length is independent of pH, temperature (15–25° C.), and the aging of the eggs.

Unfertilized and fertilized eggs are highly and equally susceptible to Na-ions. To K-ions, the unfertilized and fertilized eggs are highly resistant from 5 to 12 minutes after insemination. This indicates that in so far as K-ions in the medium are concerned the cytoplasm does not change from the unfertilized to the fertilized state until many minutes after insemination.

Indophenoloxidase in Arbacia eggs and the Nadi reaction. Albert E. Navez.

In a previous note by A. E. Navez and Ethel B. Harvey published here in 1935, measurable differences of indophenoloxidase activity of the unfertilized eggs of *Arbacia* intact, stretched and halved by centrifugalization were reported, which for equivalent volumes of material was: whole egg (1.0); white nucleated half (2.4); whole egg stretched (3.2) and red enucleated half (4.8) in arbitrary units.

In an attempt to determine the basis for the increase in activity of the stretched eggs, experimental evidence brought some doubt on the reliability of the Nadi reagent as a specific indicator for the indophenoloxidase, especially in view of the common use of indophenol blue by plant cytologists, as a stain for oleoplastic inclusions.

In an effort to detect some substance(s) reacting in the same way, whole eggs were cytolized, put immediately in contact with fat solvents (chloroform, benzene, ether, etc.), the solvent separated on the centrifuge (in closed tubes) and distilled off in vacuum at room temperature. An oily residue of high viscosity, dark red color (probably by denaturation products of echinochrome) and easily oxidized was left behind. Preliminary experiments showed also an increase in the rate of oxidation on addition of iron salts.

This "oil" (crude extract) is saponifiable (with index of saponification around 200) and fixes iodine easily (index of iodine 180–190), indicating the presence of double bonds and giving a basis for the notion of autoxidation. Both of these values are preliminary and may be corrected later on further purification of the "oil."

The most important characteristic is its reactivity with the Nadi reagent, even after warming it in vacuum for several minutes, in a boiling water bath. This, coupled with its extractability, makes the present reaction exclude the specificity of the Nadi reagent for the oxidase.

It may be inferred that this oil is actually bound to a protein when one takes into account the observation of Heilbrunn on the action of ammonium salts on the size of the oil cap in centrifuged eggs; the possibility that the unstable solution of paraphenylenediamine may contain, by secondary reactions, traces of ammonia and that on stretching eggs evidence has been found that some ammonia (or a compound able to give off ammonia) may be liberated.

The presence of an "oil" easily oxidizable brings in a new factor in the scheme suggested by Korr for the respiratory system of *Arbacia* eggs.

An oxidase determination can be made by correcting for this "oil": determinations are done successively on living cells and on killed cells (by heat).

Figures were reported respectively for the "oil" and "oxidase" parts of whole, stretched and halved cells. Evidence points towards an increase in oxidase activity in stretching of cells as well as to an increase of the reactant "oil."

Electric impedance of nerve during activity. K. C. Cole and Howard J. Curtis.

Alternating current impedance measurements have been made over a wide frequency range on the giant nerve fiber from the stellar nerve of the Woods Hole squid, *Lolego pealii*.[1] Narrow electrodes were placed on either side of the fiber, so that the current flow was perpendicular to the fiber axis. In order to observe and measure the change in transverse impedance during the passage of the nerve impulse, the amplified output of the Wheatstone bridge was applied to a cathode ray oscillograph, and as a sweep circuit moved the spot across the screen, there was a narrow horizontal trace when the bridge was balanced. When the nerve was stimulated at one end, the line broadened to a band as the impulse reached the impedance electrodes, indicating a bridge unbalance, and returned during the recovery phase.[2] By an appropriate analysis, it was found that during activity the membrane phase angle is unchanged, the membrane capacity decreases very slightly, while the membrane resistance drops from probably about 5000 ohm cm.[2] or more to an average of 25 ohm cm.[2]

The onset of the resistance change occurs somewhat after the start of the action potential, but coincides quite closely with the point of inflection on the rising phase. At this point the membrane current density reverses in direction, corresponding to a decrease of membrane electromotive force, so that this e.m.f. and the conductance are closely associated properties of the membrane, and their sudden changes are themselves, or are due to, the activity which is responsible for the propagation of the nerve impulse.

These results are qualitatively identical to those found for the single plant cell *Nitella* during activity.[3] Furthermore, the resting impedance properties of nerve fibers from several different animals have been measured [4] and found to be very similar to those of the squid fiber, so it is reasonable to suppose that similar changes occur in all nerves during excitation and conduction.

The vibratory response of the skin and its relation to pressure sensitivity. Frank A. Geldard.

Considerable confusion exists at present as to the essential nature of vibratory sensations. A widespread view is that they are mediated by a separate "vibratory sense," having its own receptors and nervous pathways and hence constituting a sense modality *sui generis*. Contrasting is the theory that vibratory senastions are but perceptual patterns of superficial touch and, as such, are but "touch in movement." Within clinical neurology the vibratory sensation is conventionally regarded to be identified with "deep" or periosteal sensitivity and the tuning fork test is an important part of the clinician's routine. The strongest evidence from clinical neurology, thus far quite uncritically evaluated, is the apparent dissociation of touch

[1] Curtis, H. J. and Cole, K. S., *J. Gen. Physiol.*, **21**: 757, 1938.

[2] Cole, K. S. and Curtis, H. J., *Nature*, **142**: 209, 1938.

[3] Cole, K. S. and Curtis, H. J., *J. Gen. Physiol.*, Sept. 20, 1938.

[4] Cole, K. S. and Curtis, H. J., Cold Spring Harbor Symposium on Quantitative Biology, **4**: 73, 1936.

and vibratory sensations in tabes, multiple sclerosis, syringomyelia, and other nervous affections.

Qualitative experiments by von Frey (1915), identifying vibratory reception with the operation of the "pressure sense," never adequately harmonized with other findings, point the way to critical quantitative determinations of the relations between vibration and pressure. On the volar surface of the arm there were located two populations of touch spots, one highly sensitive to pressure stimulation, the other quite insensitive. Controlling mechanical skin gradients, the frequency, amplitude, and wave-form of the movements of an oscillator-driven needle (Geldard and Gilmer, *J. Gen. Psychol.*, 1934), and observing skin movements by slight off-tuning of intermittent illumination in the microscope field, the vibratory thresholds of all spots were measured for the frequencies 64, 128, 256, 512, and 1024 cycles. Pressure-sensitive spots prove to have uniformly low vibratory thresholds (of the order of 25μ amplitude), while insensitive spots have high (100–250μ) thresholds and show great variability. The essential identification of vibratory and pressure sensitivities is held to be demonstrated. Further, the consequences of the widespread conduction of forced vibrations by the skin and other tissues for the interpretation of various clinical anomalies are indicated.

Reversal of phototropic reaction in Daphnia by the use of photosensitizing dyes. E. Alfred Wolf.

The property of vital stains to act as photosensitizers in light was applied to the study of phototropic reactions. Positively phototropic specimens of *Daphnia longispina* were stained in solutions of rose Bengal and eosin for three to twelve hours. Their movements in white and filtered light were observed in a glass trough covered by a black cardboard hood that left one side of the trough open to light; a slit in the top of the hood allowed observation of the animals. The animals were introduced in the middle of the trough in groups from six to nine, or singly either at positive or negative end, and their position was recorded after definite periods of time.

In a first series red and green filters of glass were used, the red absorbing those wave-lengths that would be absorbed by the stained animal itself and consequently injure the animal; the green letting those injurious rays pass that now can act on the animal. Animals were tested in groups. In red the majority accumulated in the positive half of the trough within five minutes of exposure; change to green or white caused the animals to move to the negative half, with occasional tardy specimens still in the positive half after five minutes.

In another series the path of single specimens was recorded in one-minute intervals. Liquid filters of solutions of the dyes themselves were used thus assuring complete absorption of the harmful rays; animals remained in the positive half of the trough or migrated to it if released at the negative end. After thirty minutes the filter was removed and the animals very quickly fled to the negative end. After six to twelve minutes the animals started to return to the positive end and died one to three minutes after arrival.

Motion picture showing microscopic changes in fibers of cardiac and skeletal muscle of invertebrates and vertebrates during contraction, retraction, and clotting. Carl C. Speidel. (See also abstract of paper on p. 349.)

These cinéphotomicrographs reveal many of the histological changes that accompany contraction, retraction and clotting in fibers of striated muscle. Some of the pictures were taken directly from the intact living animal; others were taken from excised tissues suitably mounted for microscopic examination. Among the activities recorded are the following: in *Palaemonetes*—contraction and folding of fibers during

electrical stimulation, the resting state followed by steady and sustained contraction of one portion of a group of fibers, and uncoördinated localized contraction nodes traveling along several fibers; in *Limulus* leg muscle—traveling contraction nodes somewhat coördinated on three adjacent fibers, collision of nodes, nodes in fibers with retraction clots; in *Limulus* heart musle—fiber showing rhythmic contraction node formation at same locus, node origin followed by travel toward one end, retraction clot barrier to contraction node advance, node formation induced by electrical stimulation, barrier of sharp bend in fiber to node advance, rhythmic node formation in heart fibers with retraction clots one day after severe heart injury, contraction node advance to a slight barrier and its division, followed by the reflection backward of one part and the advance of the other, elimination of barrier by electrical stimulation; cardiac fibers in the intact living *Polyonyx*, also in the heart of *Loligo* and *Venus*, beating lymph heart of frog tadpole, retraction clots and monocyte movements in fibers of starved tadpole, and retracting fibers of striated muscle in the swim-bladder of the sea robin.

Some of the pictures also portray the various responses to injury of fibers of cardiac and skeletal muscle of frogs, frog tadpoles, and rats, a detailed description of which has recently been published (*Anat. Rec.*, **70**, Suppl. No. 3, p. 102).

The action of direct currents on the cell nucleus (Film). W. R. Duryee.

This film has been prepared to show the movements of chromosomes and nucleoli within an interkinetic nucleus as well as to study the colloidal structure. Only in this way can the kinetic aspects and three-dimensional relationships be comprehended.

Nuclei, both isolated in Ca-free Ringer and *in situ* in transparent young cells, are from the ovarian eggs of the salamander *Triturus pyrrhogaster*. The material is mounted in 0.05 cc. drops through which 10–15 milliamperes of current from a 45-volt battery is passed.

Chromosomes and nucleoli always move toward the anode (+), reversing with the current twenty or more times, thus showing that they are negatively charged. While in the center of the nucleus they appear to move as a unit inasmuch as they are imbedded in a weak elastic gel, the Binnenkörper, which contributes to the formation of the maturation spindle. Proof of this elastic matrix is given by the fact that chromosomes and nucleoli spring back as soon as the current stops flowing, after they have been pressed against the inside of the nuclear membrane.

Similar effects are shown in intact transparent eggs. In addition, the nucleus as a whole moves toward the anode, thus indicating its negative charge, while cytoplasmic granules move toward the cathode. Darkening and contraction of the nuclear membrane toward the anode is interpreted as adsorption of cations from the cytoplasm on this side, while it may be seen that the opposite side, toward the cathode, swells and eventually dissolves due to the accumulation of anions. The latter effect is reinforced by the wave of hydroxyl ions spreading from the cathode.

Hydration and dehydration of follicle cell nuclei (Film). W. R. Duryee.

In this film kinetic relationships between environment, cytoplasm, and nucleus are demonstrated. Tangential views of the double layer of follicle cells on young *Triturus* ovocytes give both sequential data on swelling and shrinking and also cytological evidence on the structure of somatic nuclei.

Gradual dilution of the isotonic Ringer by distilled water makes the nuclei swell and become hyaline. On the other hand, shrinkage by hypertonic Ringer causes chromosome-like bodies to appear. These 20–30 presumable chromosomes are oriented vertically to the egg surface and condense more heavily on the side toward the egg cytoplasm. Since this process is freely reversible it furnishes inferential evidence on the interkinetic persistence of the chromosomes.

AUGUST 31

The function of muscles in locomotion. Herbert Elftman.

The function of the leg muscles in walking can be studied by recording the force which the foot exerts against the ground by means of an especially designed apparatus, a motion picture of the individual being taken simultaneously. From these data the torques which the muscles exert on the trunk, shank, thigh and foot can be determined and the rate at which they do work can be found. Tension is necessary in the muscles, even when the muscle tissue does no physical work, for two purposes: (1) putting other forces into equilibrium so that the desired movement is obtained; (2) transmitting energy from one part to another by "tendon" action. Muscles regulate the energy level of the locomotor mechanism by receiving energy and by liberating energy. In walking this occurs in two cycles in the muscles of each leg for each double step. This alternate reception and release of energy by the muscles suggests the possibility of energy storage. If no storage occurs, and no account is taken of other energy requirements, the efficiency of the leg muscles in dissipating energy and mobilizing it from chemical sources would be at least 39 per cent, using the figures of Atzler and Herbst for oxygen consumption during walking. The partial storage of energy by the muscles is indicated. The possibility of effective storage depends not only on the characteristics of muscle tissue, but also on the disposition of the muscles. All of the functions of the leg muscles in walking could be served by a single muscle passing over three joints. The actual muscles present represent a compromise between such a muscle, with its efficiency, and a system of one-joint muscles with greater adaptability to different types of movement.

The effects of copper and of vanadium on the frequency of division. William J. Bowen.

Chilomonas paramecium can be grown indefinitely in a solution containing only simple compounds and under environmental conditions easily and accurately controlled (Mast and Pace, 1937, *Jour. Cell. and Comp. Phys.*). It therefore offers an excellent opportunity to study the effects of the heavy metals on the growth of a protozoan.

The effects of copper, in $CuCl_2$, and of pentavalent and tetravalent vanadium, in Na_3VO_4 and $VOCl_2$ respectively, were investigated by ascertaining the rates of division during 15 consecutive days of four isolated lines of *Chilomonas* grown in culture fluid to which various amounts of the above compounds were added.

The average number of divisions of the four lines indicates that $CuCl_2$ in any concentration between 3×10^{-7} and 1×10^{-20} molar has no beneficial effect on the rate of growth of *Chilomonas*, that in a concentration of 1×10^{-10} molar it retards growth, and in a concentration of 3×10^{-7} molar it kills the organisms.

No acceleration was obtained in culture fluid containing Na_3VO_4 in concentrations ranging from 1×10^{-3} to 1×10^{-12} molar, but the organisms grew in the relatively high concentration of 1×10^{-3} molar. However, in culture fluid containing tetravalent vanadium there was definite acceleration. As the concentration of $VOCl_2$ increased, the frequency of divisions increased from 3.54 per day in zero concentration to 3.91 in 1×10^{-10} molar and then decreased.

These results with the two forms of vanadium indicate that partially oxidized vanadium is beneficial for the growth of *Chilomonas paramecium* and that the completely oxidized form is not beneficial.

Sex balance in the progeny of triploid Drosophila. Sarah Bedichek.

Males from eight X–IV translocation stocks were crossed to triploids with recessive markers, producing sterile intersexual "duplication intersexes" with a short fragment of the X plus two complete X's and three autosomes and weakly fertile

hypotriploid females with longer X fragments plus 2X3A. Duplication intersexes bearing interior regions of the X in triplicate were studied by combining right and left-hand fragments from two different translocations. Of eight duplication intersexes with very short sections, covering successively the entire X chromosome, in triplicate, none showed a marked shift in the female direction. Therefore a single primary sex gene can not exist capable of producing a functional female when represented three times with 3A regardless of the dosage of the remainder of the X chromosome. There was a graded shift toward femaleness in the phenotypic appearance of individuals with an X fragment plus 2X3A with increasingly longer fragments. Individuals were hypotriploid with either right or left-hand section of two translocations (t–lz and lz–v breaks). Furthermore, fertility of certain fragments plus 2X3A aneuploids begins before the last trace of intersexuality vanishes, since some hypotriploids with the right-hand fragment of the t–lz and lz–v breaks possess very rudimentary sex combs. For this reason and the fact that shorter sections from the left than from the right-hand region of the X chromosome plus 2X3A result in weakly functional females, we may conclude that the portion of the X to the left of the t–lz break seems a little more female potent in relation to its cytological length than the portion to the right of this break. A drop in temperature to 18° C. produces intersexuality in those individuals carrying the right-hand fragment of the t–lz break but does not alter the female appearance and function of hypotriploids bearing longer fragments.

The antihormone problems in the salamander. Eduard Uhlenhuth, James U. Thompson and Joseph E. Schenthal.[1]

In 1934 Anderson and Collip reported that after a few injections of Thyreoactivator (T.A.), the thyroid becomes refractive to this hormone, owing to the action of antihormones. Sor far we have been able to examine salamander thyroids removed after a maximum of 40 daily injections of T.A. extracted from beef A.L., but failed to observe refractoriness.

The activity of the thyroid was expressed by its colloid content calculated as "Colloid Level" (C.L.). Ringer extracts of A.L. were administered daily to adult animals of the California newt. In one experiment from 1 to 40 injections of 10 mg. of A.L., in another experiment from 2 to 33 daily injections of 50 mg. of A.L., in a third one 10, 34, 36, 38 and 40 injections of 50 mg. of A.L. were administered. In every case variations of the colloid content due to the cyclic alternation of colloid release and colloid storage are observed; but the values obtained in the beginning are not higher than those obtained at the end of the experiment. Thyroids taken after 26, 34, 36 and 38 injections have C.L. values of only 3 to 9 per cent, which are among the lowest values obtainable.

The O_2-consumption of animals of the same species, injected daily with T.A., was tested. It shows a rise while the colloid is released and a drop while it is stored, exhibiting the same wavelike variations as the colloid content but in opposite directions.

During a period of prolonged T.A. administration the thyroid is in a state of increased activity all the time, but at some time it releases colloid and at other time it manufactures and stores new colloid. No decrease in the response to T.A. was observed in the salamander up to 40 injections of T.A.

The effect of the sex-stimulating factor of the anterior pituitary gland on the testis of the bullfrog. Roberts Rugh.

Most of the maturation figures may be found in the seminiferous tubules of the bullfrog testis at any time of the year. Spermatozoa first appear at the time of metamorphosis but males are not considered sexually functional until they measure

[1] From the Department of Anatomy, University of Maryland Medical School.

about 100 mm. in body length. Since spermatozoa are present, even though there is little or no sex-stimulating hormone present in the males of body length less than 90–100 mm., it is assumed that the pituitary *growth* hormone controls maturation.

The anterior pituitary gland from mature males or females will effect marked changes in the testis of any bullfrogs, regardless of size or maturity. The procedure has been to excise and fix the left testis as the control and then to inject the frog with an adequate dose of pituitary tissue and to fix the right testis within 24–48 hours.

Since the anterior pituitary gland hormone seems to empty the testis of any loosely arranged cell products, such as the spermatozoa of the mature bullfrog and any (except the earliest) maturation figures of the immature males, it is suggested that it acts through the medium of the smooth muscle tissue so abundant in the testis. Primary spermatogonia, whether in the metamorphosing bullfrog or in the adult, are unaffected. This is probably due to the fact that they are an integral part of the marginal epithelium of the seminiferous tubule. Even the cortical pro-gonad of the prematamorphosing testis shows the effect of this hormone. It has previously been demonstrated that the anterior pituitary gland hormone not only causes the rupture of the ovarian follicle but stimulates the smooth muscle cells of the follicle wall to force the egg out through the rupture area. Further evidence of the activation of this smooth muscle tissue is the fact that if an adult testis is studied within 16–20 hours after the host has been treated with the hormone, only the peripheral seminiferous tubules will have been emptied. Final confirmation of this thesis will require the severing of the sympathetic connections with the gonad prior to anterior pituitary hormone treatment.

Studies on barnacle larvae. J. Paul Visscher.

Alcippe lampas is a boring barnacle which inhabits mollusk shells which are occupied by hermit crabs. It has been found in 45 per cent of the shells examined and one shell had 37 specimens in it. The animal is found completely embedded in a cavity in the shell. This cavity has a relatively small orifice through which the appendages of the barnacle are thrust for feeding and for respiration. The barnacle may attain a diameter up to one-half inch. It is disk-shaped and is usually 3 or 4 times greater in width than it is in thickness.

The larvae of this barnacle were found abundantly in June and but rarely in late July and August.

The eggs of this animal are large and full of yolk and the nauplii were observed to moult regularly without feeding. There are six nauplear stages—each persisting for about 24 hours under the conditions of the experiment. The cyprid larva is long, slender and very active. The activity is noticeably increased by the addition of a fluid extract of the tissues of the hermit crab.

Conclusions:

1. *Alcippe lampas* is a common barnacle in the Woods Hole region.
2. The nauplii pass through six stages or moults in about one week.
3. The nauplii do not feed during this period.
4. The cyprid larvae are activated by the presence of the hermit crab, *Pagurus policarus.*

The spawning reaction of Nereis limbata with emphasis upon chemical stimulation. Grace Townsend.

Alternation of light and dark can induce a corresponding alternation of swimming and quiescence suggesting that occurring in nature. With sufficiently strong light to induce rapid quiescence, this can be repeated at half-hour intervals. Tactile stimulation is necessary for achieving the quiescent state. Without a bottom layer of sand or provision for tactile stimuli, continuous swimming occurs in any illumination.

A vibration sense localized on the parapodial cirri is sensitized during sexual metamorphosis and enables the swimming worms to avoid collision. This sense is supplementary to the chemical sense in inducing circling orientation and under certain conditions may induce circling independent of chemical stimulation.

The response of the male is a typical sensory response and normally occurs only during stimulation. The female response possesses neuro-hormonal characteristics and may continue in the absence of stimulation. The male response may sometimes be artificially induced to resemble the female response.

A special sensitivity to egg-water, cystine, cysteine, and glutathione, not existing in the non-sexual phase, is developed on the epitoke of the male at sexual maturity. The sensitivity appears to be of great chemical specificity, extending even to the distinction of dextro and laevo forms of cystine.

A physiological completion of sexual metamorphosis occurs during swimming. The quantity of male-stimulating material is increased in the female and the sensitivity of both males and females to material of the opposite sex is increased. The processes involved are reversible.

Physiological assays concerning the nature of fertilizin. Grace Townsend.

The male of *Nereis limbata* has been hypothesized by Lillie and Just (1913) to spawn in response to material from the egg which is neutralized by the sperm and is essential to fertilization. *N. limbata* males are sensitive to 1 : 1,000,000 parts glutathione. Contrastingly they do not spawn in response to unrelated sulphydryl compounds, various amino acids, iodides, hormones, enzymes, histamine, acetyl choline, and miscellaneous compounds tested. Marine eggs contain from 0.03 to 0.04 per cent glutathione wet weight. A reducing substance as tested by the very sensitive ortho-chloro-phenyl-indophenol leaches from the eggs.

The material of egg-water and glutathione possesses all investigated properties in common: location in the egg, solubility in water, precipitation by acetone, stability in acid, lability in alkali, dialyzability, destruction by aging, boiling, irradiation, copper, arsenic, and monoiodacetic acid, lack of destruction by NaCN, adsorption by kaolin and charcoal, apparent inactivation by sperm, blood, and cytolyzed egg, increased physiological potency in the induction of spawning in a slightly acid medium. These properties, in so far as covered by the studies of Woodward (1918) and Glazer (1921), are in agreement with those ascribed to the parthenogenetic fraction of "fertilizin." They differ from the properties assigned to the agglutinin fraction and from the properties assigned to "fertilizin" by Lillie (1913) with respect to dialyzability and degree of lability in the presence of heat and upon aging. For a given molecular concentration, the material of egg-water is more potent than glutathione.

The fertilizability of washed eggs is partially restored by immersing the eggs in 1 : 3–5,000 parts glutathione.

A study of the integument of the squid, during staining with methylene blue. Elbert C. Cole.

The selectivity of methylene blue for various tissue elements was found to be greatly increased by the addition of appropriate substances to the staining solution. A living squid was placed in a screw-cap jar and the jar nearly or quite filled with a standard solution of one part of certified methylene blue in 20,000 parts of sea water. The jar was sealed and staining under anaerobic conditions allowed to proceed for an hour or more. Upon exposure of the squid to air, nervous elements of the integument were found to be stained. The addition of 1 per cent of methenamine (the brand "Urotropin" was used) to the standard solution increased selectivity for nervous tissue. The addition of KCN, one part in 200,000, together with ethyl urethane, one part in 300, to the standard solution produced highly selective staining of the radial muscle of the chromatophores.

With these solutions a study of the structure and activities of the various components of the integument was made. The action of the radial muscles during movement of the chromatophores, the innervation of the chromatophores, the attachment of radial muscles to the connective tissue framework of the skin, and the stresses and strains produced on this framework by violent contractions of radial muscles were studied. It is suggested that these methods may be generally applicable to the study of structure and function of particular cells in complex tissue masses.

The use of the clam heart as a test object for acetylcholine. Carl C. Smith and Louis Levin.

A method is described for the use of the heart of the marine clam, *Venus mercenaria*, as a test object for acetylcholine. Details of the procedure for isolating the heart are given and a special chamber adapted to the purpose is described.

The sensitivity of the heart to choline was studied and the smallest concentration found to give a demonstrable inhibition was 1 : 50,000. Acetylcholine in a concentration of 1 : 1,000,000,000 produced a 50 per cent reduction in amplitude of the beat from which the heart recovered quickly. In solutions containing choline and acetylcholine, in which the choline is present in a subminimal quantity, no effect upon the normal inhibition produced by acetylcholine was observed. Sensitization by eserine is not needed. With this preparation in the apparatus described, the least amount of choline which can be detected is 0.2 mg.; the smallest amount of acetylcholine which can be assayed is 0.00001 mg. or 0.01 gamma.

The fact that the clams are easily available and can be kept for long periods makes the use of this preparation advantageous as a simple tool for the bioassay of choline derivatives.

The elimination of molds.[1] Oscar W. Richards and Katherine J. Hawley.

Eighteen different species of mold grow profusely in the Chemical Room of the Marine Biological Laboratory during the summer when the relative humidity exceeds 80 per cent. The molds grow on almost everything except glass and metal and during the last five years experiments have been made to eliminate the mold growth in order to prevent damage to equipment and materials. Twenty-one chemicals were tested and most of them were ineffective under the severe condition encountered. The most promising fungicides are thymol, cinnamon oil, α-nitronaphthalene and Phenox. Seasonal protection of most materials is possible with these chemicals and concentrations are recommended for their use. Longer protection may come by combining these or more potent chemicals with a suitable binding varnish which will hold an effective concentration of the fungicide on the surface of the material to be protected.

A comparative study of water aspirators. S. E. Pond, E. P. Little, A. M. Smith and J. D. Graham.

Devices in use for simple evacuation and for air lifting, by means of water pressure, vary considerably: (*a*) final vacuum, (*b*) air volume moved, or speed of evacuation, and (*c*) water consumed in operation. Some units have been found to take larger quantities of water for effective operation than appears necessary and are therefore unusually costly for general use. In tests reported, a two-liter flask was evacuated, with water operated at 40 lbs. per square inch, permitting a group classification of the aspirators which were available:

I. Vacuum established and maintained in 60 seconds.

[1] The detailed report of this work has been accepted for publication in the *Jour. of Chem. Educ.*

II. Maximum vacuum reached in time indicated, with water consumption (expressed as cubic feet per minute).

III. Effectiveness with an arbitrary but uniform aperture (air intake) during operation, indicated by column of mercury in cms. lifted during operation, at equilibrium.

Twelve different aspirators were studied representing seven different makes:

Group No.	Vacuum 60 sec.	Water cu. ft. / min.	Final Vac. Cm.	Final Vac. Time	Lift cm.	Composition
A 1.....	74.2	0.69	74.2	55″	33.0	Stainless steel
2.....	72.0	0.54	74.2	2′	15.0	Brass
3.....	71.5	0.45	73.8	2′	20.0	Brass
B 4.....	64.5	0.21	74.3	5′	9.3	Stainless steel
5.....	61.5	0.27	74.2	5′	6.6	Brass
6.....	61.0	0.26	72.5	5′	10.3	Brass
C 7.....	59.5	0.57	69.3	5′	5.1	Stainless steel
D 8.....	57.1	0.24	72.7	7′	10.1	Glass
9.....	54.8	0.17	73.7	7′	4.7	Brass
10.....	50.5	0.26	72.2	7′	6.9	Glass
11.....	48.0	0.21	74.0	7′	4.3	Glass
12.....	48.2	0.21	72.9	5′	4.9	Brass

The first two groups differ appreciably in speed of operation and markedly in water consumption; but group "C" is of still slower speed with very high water consumption. Pump No. 1 is the largest obtainable, essentially for air pressure work where motor-driven pumps are not available and would be used where extreme speed is required for water power. The last group, "D," indicates the most economical for moderate speed under the conditions of the tests, and includes the glass pumps which appear most useful for handling corrosive vapors when insufficiently trapped.

All pumps have been operated with the check valves as supplied in spite of the fact that these corrode easily and have an effect upon speed of evacuation. Other points of design, related to effectiveness in operation, are under consideration.

Papers Read by Title

The effect of electric current on the physical consistency of sea urchin eggs. C. A. Angerer.

This investigation is one of a series undertaken to show the effect of various stimulating agents on protoplasm. These experiments have been made primarily with the view of continuing the study on protoplasm of some phylogenetic different species from that used heretofore, namely, Amoeba. The physical consistency was determined by the centrifuge method. The material used was the unfertilized eggs of the sea urchin (*Arbacia punctulata*). The stimulating agent was electric current (direct and alternating). The eggs were subjected to electric current through a $Zn/ZnSO_4$/sea water-in-agar system which in turn was in circuit with either the A.C. or D.C. service line running into the laboratory. Relatively strong current densities had to be employed in order to affect noticeably the physical consistency of the eggs. A current density of 0.005 amperes/cm.^{2} cross-sectional area of available electrode

surface was employed for both alternating and direct current. When plotting the relative "viscosity" as a function of varying intervals of time (in seconds) during which any given quantity of eggs are exposed to the current density in question, there is during the first 2 seconds of current flow a progressive decrease in "viscosity." With continued application of the current the "viscosity" levels at a minimum which is 66 per cent of the control value. With still further application of the current the "viscosity" begins to increase after 5 seconds of exposure at the linear rate of approximately 10 arbitrary units/second. The experiments were discontinued after 15 seconds. The experimentally induced changes in "viscosity" are reversible and on insemination of the eggs normal membrane elevation and cleavage occur. These results on electrical stimulation are in line with those already obtained for the action of this and other stimulating agents (mechanical agitation, heat and ultra violet radiations) on Amoeba and sea-urchin egg protoplasm.

The effect of sodium azide on the frequency of the embryonic Fundulus heart. C. W. J. Armstrong and K. C. Fisher.

Recently Keilin (*Proc. Roy. Soc.*, B, **121**: 165, 1936) has shown that many of the effects of cyanide in cell respiration are duplicated by sodium azide. Since it has been shown that the effect of cyanide on heart-beat frequency in embryonic fish can be quantitatively characterized, it is of interest to use the heart-beat frequency as a quantitative test of how far the similarity between cyanide and azide extends.

Eggs of *F. heteroclitus* (from 3 to 7 days after fertilization) were irrigated with aqueous solutions of sodium azide of concentrations ranging from .004M to .00002M. The solutions were approximately neutralized (phenol red) to a pH of about 7.2 by the addition of a few drops of .1N NaOH. Normal frequencies were obtained by observing 12 to 16 organisms in a tube in running distilled water. The distilled water was then replaced with azide solution and the consequent frequency decline observed until a new frequency level had been attained (= inhibition level). Occasionally this level was observed to persist for many hours. The maximal inhibition level (= 47 per cent normal frequency) was obtained with solutions of a concentration greater than .001M.

By plotting log [azide] against log $\frac{\text{per cent inhibited frequency}}{\text{per cent uninhibited frequency}}$ (where per cent inhibited + per cent uninhibited frequency + maximal inhibition level = 100 per cent of normal frequency) a linear relation between the two appears indicating that the data can be described by the Mass Law.

The degree of inhibition depends upon the pH. The following results were obtained using a .0001M sodium azide solution.

pH	Inhibition Level (as per cent normal)
8.0	100
7.3	80
6.0	50

Cf. the effect on yeast etc. (Keilin, 1936).

A comparison of the effect of azide on heart-beat frequency in *Fundulus* with that of cyanide (the latter from the work of K. C. Fisher and R. Öhnell) is given.

	Azide	Cyanide
1. Maximal inhibition level (M.I.L.)	47	27
2. Weakest solution to produce (M.I.L.)	.001M	.001M
3. Time to attain M.I.L. using 2	5–7 hrs.	4 hrs.
4. Slope (n) of line from log plot	1.3(5)	1.6

Reducing activity of fertilized and unfertilized Arbacia eggs. Robert Ballantine.

The ability of *Arbacia punctulata* eggs, fertilized and unfertilized, to reduce methylene blue (also cresyl blue and toluene blue) has been studied under comparable conditions in a nitrogen atmosphere and the results expressed as the mm. of the oxygen equivalent of methylene blue reduced per hour per mg. N_2 in the eggs. On the average, 1 cc. *Arbacia* eggs contains 28.5 mg. N_2. Fertilized eggs reduced methylene blue 233 per cent to 93 per cent more rapidly.

The reduction rate does not change from 10 minutes to 1½ hours after fertilization and the sperm which are used to fertilize the eggs are not responsible for the increased reduction time: neither do fertilized or unfertilized eggs give off anything to the medium which has reducing power. Blocking iron systems with Na azide does not affect reducing time in either fertilized or unfertilized eggs.

Addition of substrate (e.g. lactate) to either fertilized or unfertilized eggs does not change reducing time, presumably because lactate does not penetrate. If cytolyzed in 50 per cent sea water without washing, reduction is much more rapid, especially in unfertilized eggs. If cytolyzed and washed, reduction is very slow but on adding lactate reduction increases more than control uncytolyzed eggs, whether fertilized or unfertilized.

Rate of absorption of both methylene blue and cresyl blue by unfertilized and fertilized eggs is the same under aerobic conditions. Methylene blue penetrates much less rapidly aerobically than it can be reduced in the medium under unaerobic conditions. The change to leuco dye within the cell must maintain a very steep diffusion gradient.

Study of per cent reduction (determined electrometrically)—time curves indicates that permeability to dye cannot entirely explain the greater reducing time of fertilized eggs but that their behavior may be connected with increase of available substrate or of active dehydrogenases.

Studies on the mechanism of growth in Planaria maculata. Ludwig von Bertalanffy.

In continuation of a quantitative theory of growth, as developed in former investigations of the author (cf., for instance, *Human Biology*, **10**: p. 181ff.), some basic factors involved in growth have been investigated in *Planaria maculata*. As suggested by Hesse and others, and quantitatively formulated in the author's theory, it may be assumed that growth is controlled by the fact that in the beginning the magnitude of resorbing surfaces, and, therefore, the quantity of building material per unit mass is relatively high; but that the latter becomes gradually smaller due to the fact that the relative size of interior surfaces decreases, leading thus to a decrease of growth rate and finally to an equilibrium in which the resorbed materials only maintain the balance, and growth stops. This hypothesis has been tested, for the first time, in a quantitative way. Measurements of the *magnitude of the surface of the intestinal tract* have been made by various methods. It was found that: (1) the length of the intestinal tube increases many times with increasing size of the body; (2) that the same holds for the surface, but that (3) the relations of length of the intestinal tract: weight, and interior surface : weight continually decrease; (4) the relation of the magnitude of interior surface: weight $\frac{2}{3}$ remains approximately constant over three orders of magnitude, some hundredths of a milligram in newly-hatched individuals to some twenty mg. in full-grown specimens. This indicates that, though there is a considerable enlargement and branching in the intestinal tract, nevertheless the interior surface behaves as if it were the surface of a proportionally growing body, controlling growth in the way mentioned above. Further experiments have been done in determining quantitatively *starvation*, *temperature influence*, *metabolism*. In higher temperatures, occasional *monsters* and *duplicitates*, resembling the results of ex-

perimental injuries, are found among hatching animals. Full descriptions of methods and results will be given in another place.

Probable absence of a chromatophore activator in Limulus polyphemus. Ruth Snyder Cooper.

Hosoi (1934) reported a chromatophore activator in several organs of *Penaeus*; furthermore, Hanström (1937) found that heads, but not eyestalks, of *Hippa* and *Gebia*, and heads and thoraces of certain insects possess chromatophore activators. It therefore seemed of interest to test organs of the arachnoid *Limulus* for a chromatophore activator.

Sea water extracts were made of lateral eyes, plus some adherent nerve fibers, removed from living adult *Limulus*. Cephalothoracic and abdominal injections of such extracts into black-adapted, white-adapted, and blinded *Palaemonetes* induced no chromatophoric contractions. Survival being highest following abdominal injections, all subsequent injections were abdominal.

After killing adult *Limulus* in 80° C. sea water (5 minutes), organs were immediately removed and stored at 4.5° C. until extraction. Diluted blood (1 : 1.66 sea water) of such freshly killed *Limulus* effected no chromatophoric responses of black-adapted or blinded *Palaemonetes*; undiluted blood was lethal. Similar organs of ten *Limulus* were mashed together and extracted in at most 2½ cc. of sea water at 100° C. for ten minutes. Filtered extracts of "brains," thoracic plus abdominal nerve cords, portions of male gonad and gastro-hepatic glands, and nephridia were injected into black-adapted, white-adapted, and blinded *Palaemonetes*. Survival of *Palaemonetes* after injection was as follows: for eye—100 per cent, gonad plus gastro-hepatic gland—100 per cent, nephridia—66 per cent, blood—66 per cent, nerve cord—55 per cent, and brain—20 per cent. All surviving animals failed to show chromatophoric responses.

The experiments were controlled by sea water and *Palaemonetes* eyestalk extract injections into *Palaemonetes*, as well as by tests of adaptability to white or black backgrounds. Although but 50 test *Palaemonetes* were employed, it appears that the above organs of *Limulus* are devoid of a chromatophore-activating principle.

Intracellular pH studies on the ova of Mactra solidissima. C. G. Grand.

The germinal vesicle of the *Mactra* egg occupies a large part of the cell. It persists for hours and does not break down normally until after insemination of the egg.

The intracellular pH and acid of injury of *Mactra* eggs in the germinal vesicle stage were determined by the micrurgical technique. The determinations of pH were made colorimetrically using 0.4 per cent aqueous solutions of phenol sulphon-phthalein indicators. The indicators used were: cresol red, phenol red, brom cresol purple, chlor phenol red and brom cresol green.

None of these indicators served as vital stains in ordinary immersion methods except when the cells were dead. Dead cells stain intensively with most of these indicators, the pH reaction of the cytolyzed material being the same as that of sea water. This fact made it possible to determine those cases in which the microinjections were performed without injury.

The eggs were operated upon while immersed either in normal alkaline (pH 8.4) or acidified (pH 5.6) sea water. This procedure was used to check against the possibility that cytoplasmic pH was influenced by the surrounding sea water.

Cresol red, phenol red and brom cresol purple were injected into the cytoplasm of the eggs. The diffuse coloration resulting from the injections was as follows: cresol red—*yellow*, phenol red—*yellow orange* and brom cresol purple—*blue*. Similar injections into the germinal vesicle gave the following colors: cresol red—*pinkish yellow* (near its acid range) and phenol red—*red* (alkaline).

The action of fixatives on the isolated cell nucleus (Film). W. R. Duryee.

When a nucleus, such as the germinal vesicle of an amphibian egg, has been divested of its coverings of cell wall and cytoplasm, it becomes especially suitable for the most precise observations. The film records the process of normal nuclear fixation with Bouin's, Zenker's and Carnoy's fluids, showing that in each case the hydrogen ions penetrate first, reversing the sign of the charge on the nuclear colloids, and that the other components enter later, causing secondary precipitations, displacement of the chromosomes, and shrinkage of 60 to 79 per cent in volume. It is demonstrated that gelatinous material is extruded through the nuclear membrane during shrinkage. Such material may give rise to the "shrinkage space" seen in sections of ovocytes and should not be confused with cytoplasm.

Superimposing Zenker's fluid on Carnoy's produces an entirely different picture from the latter alone. Consequently the fixed image is neither permanent nor unalterable.

Stretching, tearing and dislocation of the chromosomes are shown. Of particular significance is the fact that the swelling of the colloidal matrix due to the primary penetration of hydrogen ions may result in liquefaction, allowing the chromosomes to sink to the inner surface of the nuclear membrane before secondary coagulation sets in. Such chromosomes are unnoticeable in sectioned material.

Weak fixation with 0.001 molar $CaCl_2$ is reversible upon addition of 0.003 molar NaOH. Successive reversals with increasing strengths of acids and bases are demonstrated.

It is concluded that none of these fixatives can yield a valid picture of the nucleus.

The melanophore-expanding activity of the ascidian neural gland. Adolph Elwyn.

Tests conducted with the neural glands of *Molgula manhattensis* and *Molgula occidentalis* definitely indicate the presence of a substance capable of effecting pigmentary changes. Saline suspensions of pulverized acetone-dried glands of *M. manhattensis* when injected into the anterior lymph sac of *Rana pipiens* always gave positive reactions. The pale or partly pale frogs began to darken in about thirty or forty minutes, reached a certain maximum which was maintained for a variable period and slowly regained their initial color value, the whole reaction lasting between two and four hours. Injection of similar quantities of other *Molgula* tissue or of normal salt solution produced no observable changes. The introduction of twenty-five freshly mashed glands likewise produced a noticeable darkening though not as marked as with the dried material, which was used in larger quantities.

Most striking was the effect in tadpoles of *Rana catesbiana*. In four tests all the animals injected with dried gland suspensions showed intense darkening, beginning in a few minutes and lasting a variable time, one over five hours, the others from two to four hours. Again the injection of other *Molgula* tissue produced no appreciable change.

In *Fundulus heteroclitus* the changes were much more irregular and difficult to interpret. In four cases there was only a local darkening varying in extent, in the region of injection, often supplemented by the appearance of small dark patches in other regions of the body. These results had to be completely discounted since similar changes could be produced by other *Molgula* tissue or even by normal saline alone. Only in three experiments were the results positive, the local reaction being gradually followed by a uniform darkening of the whole body, which was maintained for several hours. Further tests will have to be made.

Positive results were likewise obtained from the freshly dried neural glands of the larger Florida form, *Molgula occidentalis*, collected in 1937. The test animals were *Rana pipiens*, *Anolis carolinensis* and *Hyla cinerea*. It was found, however, that glands which had been kept in the dry condition for four months or longer had apparently lost their capacity for producing pigmentary changes.

Cardiac rhythm in Pecten irradians (Lamarck). Richard W. Foster, John D. Crawford and Albert E. Navez.

The frequency of heart-beat of *Pecten irradians*, for the heart in situ and excised, has been studied in relation to its autonomism and its dependence on other organs.

In situ the frequency is determined by some characteristic proper to each individual heart, which can be modified (mostly reduced even to complete block) by movements of the valves, gills or parts of the mantle.

The absolute value of the rate depends on the size of the animal among other factors. A 5-cm. *Pecten* under optimum conditions has a heart rate of 35 beats per minute at 22–23°.

The excised heart has a frequency of contraction decidedly lower than the in situ heart, depending on the portions of the heart excised.

Its frequency may range from about 27 beats per minute to values as low as 8 beats per minute. It is very sensitive to water circulation, aeration, temperature and stimulation, the amplitude of the beat being specially sensitive to temperature.

The nucleus, when shelled out by tearing the egg into acidified sea-water containing phenol red, first turned red (alkaline) and then shifted rapidly to the yellow color (acid) of the surrounding medium.

Mechanically injuring the eggs produced an acid of injury, the pH of which was determined by observing the color changes of previously injected indicators. Before cytolysis, brom cresol purple was blue (alkaline) and phenol red was yellow (acid), representing a pH of approximately 6.8. Immediately following cytolysis these indicators changed to the color of their acid range, viz., yellow. The lower pH range of the acid of injury was determined by using brom cresol green which gave the blue of its alkaline range.

In conclusion, the pH of *Mactra* eggs was found to be as follows: Cytoplasm, 6.8 ± 0.2; nucleus (germinal vesicle), 7.4 ± 0.2. Mechanical injury with cytolysis gave the acid of injury of pH 5.4 ± 0.2. These findings are in accordance with those of other investigators who obtained similar pH values from studies on other types of cells.

The alarm reaction and adaptation syndrome in lower vertebrates (Fundulus majalis). Stephen Karády.

When adult *Fundulus majalis* and *heteroclitus* are given intraperitoneal injections of sublethal doses of various injurious agents (histamine, adrenalin, pituitrin, atropine), their reaction to the first dose is always the same, being independent of the nature of the drug. The syndrome consists of haemorrhages into the intestinal tract, hypertrophy of the islands of Langerhans, fatty degeneration of the liver, etc. Such changes constitute the response of the organism to injury as such, and can be termed the alarm reaction.

These pathological changes disappear in 24–48 hours. Thereafter the resistance of the fish is increased, not only to the same drug which produced the reaction but also to the others as well. The fish can now tolerate doses which ordinarily are lethal. In like manner the color changes produced in the skin are less intense after the first injection. This condition is called the stage of non-specific resistance.

If the treatment with any one of these drugs is continued for a longer time (8–10 days) the resistance of the organisms to that particular agent increases continuously, no signs of the alarm reaction appearing. But the resistance to the drugs other than the one used disappears (stage of specific resistance).

If the treatment is still further continued, the specific resistance acquired during this stage is gradually lost, and the fish die with symptoms similar to those observed during the first stage. This last condition is called the stage of exhaustion.

Micro-estimation of protein adsorption at oil-protoplasm interfaces. M. J. Kopac.

The adsorption of proteins at oil-protoplasm interfaces has been demonstrated by studying the Devaux effect [Kopac, this number]. Frequently a spontaneous Devaux effect cannot be obtained on oil-drops injected into starfish eggs several minutes after cytolysis. A method has been developed for showing that protein adsorption, nevertheless, occurs under these conditions.

If continuity between an oil-drop and a micropipette is maintained, the oil may be slowly drawn back into the pipette. The area of the interface is reduced by decreasing the diameter of the oil-drop. Where the oil is in contact with aqueous phases lacking protein, the diminishing oil-drop remains perfectly spherical until it is entirely drawn into the micropipette. If, on the other hand, the oil has been in contact with an aqueous phase containing protein, and then slowly aspirated into the micropipette, the sphericity is maintained only to a critical diameter. A further decrease in diameter causes the appearance of the Devaux effect. The wrinkling which occurs at this critical diameter, according to the studies of Devaux and Langmuir, is due to a monolayer of protein completely covering the oil at the interface. The adsorbed protein is concentrated as the area of the interface is decreased until the monolayer coated condition obtains.

The ratio, δ, which equals $\pi d_c^2/\pi d_o^2$, may be used as an index to indicate the amount of protein adsorption. Here d_o represents the original diameter of an oil-drop in contact with the aqueous phase and d_c is the critical diameter at which the Devaux effect appears. If $\delta = 0$, no adsorption has occurred. If $\delta = 1$, the Devaux effect appears spontaneously. With $\delta < 1$, protein adsorption is indicated, the amount of adsorption being directly proportional to the value of δ. In starfish oocytes cytolyzed for a few minutes, the δ-values are less than 1 but, in many cases, the values approach 0.9, indicating that appreciable amounts of protein have been adsorbed although insufficient to produce a monolayer coating on the original drops.

Accumulative adsorption of protein can be demonstrated by expanding the same oil drop and then decreasing its diameter. If the new critical diameter is the same as before, no additional adsorption occurred. However, an increase in critical diameter indicates accumulative adsorption. Accordingly, rates of adsorption may be obtained by combining critical diameter measurements with time.

Extremely small amounts of protein can be estimated. For example, an oil-drop, 10 microns in diameter, has a surface area of 3.14×10^{-6} cm^2. By taking Philippi's value of 1.15 m^2/mg. for egg albumin, a monolayer of protein at such surface would correspond to 3×10^{-12} g. On the basis of a molecular weight of 35,000, this weight of protein represents approximately 5×10^6 molecules.

Effect of the vitelline membrane on coalescence of Arbacia eggs with oil-drops. M. J. Kopac and Robert Chambers.

Various marine eggs, if denuded of their jelly layer and tough extraneous membranes, will coalesce with oil-drops [*J. Cell. Comp. Physiol.*, 9: 331; 345, 1937]. An oil-drop flowing from a micropipette and in contact with the cell-surface is expanded at a constant rate until coalescence occurs. There is a critical diameter (d) of the oil-drop below which the cell will not coalesce with the oil. Coalescence does occur if the diameter exceeds the critical value. The resulting potential energy change (ΔE) is approximately equal to the surface area (πd^2) of the drop times the difference in tension (ΔT) at the surface of the drop before and after coalescence, namely, $\Delta E = \pi d^2 \Delta T$.

The critical diameter and accordingly the ΔE indicate the presence of a potential hill at the cell-surface. By determining the critical diameter of a coalescing oil-drop it is possible to calculate the magnitude of the potential hill. This value may be used as an index to denote the tangential rigidity, inter alia, of the cell-surface. In these

experiments the potential hill of eggs under experimental treatment was compared with that of normal, unfertilized eggs, and this ratio is represented by δ in the table.

Cottonseed oil with a ΔT-value of approximately 10 dynes per cm. was used. The *urea-eggs* were unfertilized eggs immersed in 1 M urea for 2 minutes and returned to sea water. The fertilization membranes were removed from inseminated eggs by shaking. The critical diameters, d, are the most probable values under a given experimental treatment while the d_o values are those of oil-drops with which normal, unfertilized eggs coalesced.

Treatment of Eggs	Age of Eggs Hours	Critical Diameters		$\delta = \frac{\pi d^3 \Delta T}{\pi d_o^3 \Delta T} = \frac{d^3}{d_o^3}$
		d_o	d	
Control	1–2	97 μ	97 μ	1.00
Fertilized, membranes removed	<1	77	23	0.09
	<1	92	25	0.07
Immersed in 1 M urea, 2 minutes	<2	97	32	0.11
	4	97	37	0.15
	7–8	82	28	0.12
Churned with microneedles	2	97	40	0.17
	8	82	35	0.18
Aged in sea water	8		82	0.71
	9–10		62	0.42
	12	97	44	0.20
	15–16		26	0.07
	20		9	0.01

Fertilized eggs yield low δ-values. In these eggs the vitelline membrane has been transformed following insemination to a fertilization membrane and subsequently removed by shaking. This result shows that the vitelline membrane is a significant barrier (high potential hill) to coalescence. Eggs which were churned and eggs treated with urea (no fertilization membrane develops following insemination) have correspondingly low δ-values. Here the coalescence data indicate that the vitelline membrane is either mechanically destroyed or chemically dispersed. Likewise, on the basis of decreasing δ-values, one concludes that the vitelline membrane gradually disintegrates in aging, unfertilized *Arbacia* eggs.

The osmotic pressure of gum acacia solutions. George Saslow.

In 1934 Dodds and Haines reported that the osmotic pressure of 6 per cent acacia solutions was reduced by 0.9 per cent NaCl to a value about half the colloid osmotic pressure of mammalian serum. The successful use of acacia solutions in total plasmapheresis of dogs (Amberson and co-workers) and measurements of the osmotic pressure of acacia solutions used in frog perfusions (Saslow) suggested that the measurements of Dodds and Haines be repeated.

The osmotic pressures were measured with the Krogh and Nakazawa osmometer; a precision of -8 mm. H_2O in 250 mm. was attainable. Three different samples of acacia were used. All were preparations of the Lilly Research Laboratories.

The results were as follows. NaCl added to 6 per cent acacia reduces the colloid osmotic pressure, the more so the greater the final salt concentration. The colloid osmotic pressure of the three samples, in 0.9 per cent NaCl, was 262, 250 and 255 mm. H_2O at 20°. The osmotic pressure of one sample in buffered Ringer-Locke's was 248 mm. The measurements agree well with those of others on material from the same source (Wies and Peters, Turner).

Since the colloid osmotic pressure of human serum containing 6.5 per cent protein was found to be 256 mm. H_2O (Wies and Peters), it is clear that the conclusions of Dodds and Haines do not apply to acacia prepared according to the procedure of the Lilly Research Laboratories.

Differences between Scottish and American amebas of the species Chaos chaos Müller. A. A. Schaeffer.

The accounts describing nuclear division in the common laboratory ameba, *Chaos diffluens*, vary widely, as is well known. Thus, for example, Taylor, Carter and their co-workers in Scotland report the typical occurrence of an irregular type of mitosis that might perhaps be better described as amitosis; but American investigators, on the other hand, report division as definitely mitotic. On the assumption that there is no genetic difference between the Scottish and the American amebas, the diversity in the accounts setting forth the details of nuclear division has in consequence been laid to faulty technique or to the use of pathological material. It appears now, however, that the reason for this conclusion is more complicated and may even be unjustified.

A culture of amebas obtained through the kindness of Monica Taylor of Dowanhill, Glasgow, Scotland has been cultured in Woods Hole with the same culture methods as are used to grow American amebas, and a preliminary survey of the characteristics of the two strains shows that the *Chaos diffluens* of Scotland is distinctly and consistently different from that of America.

The main differences are as follows:

(1) The Scottish amebas have fewer ectoplasmic ridges than the American amebas.

(2) The cytoplasmic division stage—moruloid—is typically a freely rolling ball; the American ameba at this stage is firmly attached to the substrate.

(3) Nearly all nuclei of the Scottish amebas are irregularly folded; this condition is extremely rare in the American strain.

(4) If the Scottish amebas are agitated when in that part of the moruloid stage corresponding to late anaphase, a very distinctive shape is taken on by the ameba. The main mass of protoplasm is a smooth sphere or ovoid with a tuft of pseudopods at either end; the American ameba does not respond to agitation in this way.

(5) Only a few permanent preparations of nuclear division stages have been made, but these seem to bear out Taylor's conclusion that mitosis is very irregular.

(6) The Scottish amebas grow as fast, if not faster than the American strain, under the same cultural conditions and appear to be quite as hardy.

There has been no opportunity so far to observe the formation of agametes which Taylor describes.

These two strains undoubtedly belong to the same taxonomic species so far as the general morphology of the vegetative stage is concerned. The difference between the two strains may be genetical or it may be due to a submicroscopic parasite or symbiont. Only further work can supply the answer.

The Scottish strain is apparently not confined to Scotland, for this strain or variants of it, have been reported from other countries of Europe by various investigators since 1867, when von Stein first described a multinucleate *Chaos diffluens*.

Induction in Griffithsia. Victor Schechter.

In following up work done several years ago on the effect of electric current (*Jour. Gen. Physiol.*, **18**, no. 1) and the centrifuge (*Biol Bull.*, **68**, no. 2) on the induc-

tion of shoots and rhizoids in *Griffithsia* cells, a preliminary mapping has been made of the effects of butyric acid, indolebutyric acid and *a*-naphthalene acetic acid. The use of hypertonic sea water and butyric acid as in artificial parthenogenesis of egg cells has been largely lethal in effect but, when the cells survived, a greater number of shoots were induced. When material was allowed to develop in 0.4 per cent and 0.04 per cent sea water solutions of N/10 butyric acid, a stimulation in number of shoots formed was indicated. *a*-Naphthalene acetic acid showed a stimulating effect on shoots in 0.00001 per cent sea water solution. The best results were obtained with indolebutyric acid in 0.01 per cent sea water solution. Here the shoots were much larger and more numerous than in controls developing in sea water alone.

These results are tentative and together with effects on rhizoids must be quantitatively evaluated.

Bacteria in relation to longevity of egg cells. Victor Schechter.

In a study of the longevity of egg cells the influence of bacteria must be taken into account. It has been found that paper-filtered sea water in which *Mactra* eggs have aged and died is extremely toxic to fresh eggs. Toxicity may be in good part removed by pasteurization at 60–80° C. or by Berkefeld filtration. These methods do not show conclusively whether bacteria themselves, or whether large colloidal particles of disintegrated eggs, are responsible for the lethal effect; the Berkefeld filter would remove larger colloidal particles as well as living bacteria and heat would denature proteinaceous toxins as well as kill bacteria. The use of the bactericide "Merthiolate" seems to have a beneficial influence if added early to an egg culture but none on fresh egg cells added to an aged culture. This may prove, if substantiated, that egg disintegration products kill other eggs and that the action of bacteria is not directly on living eggs. The results so far are clouded, however, because "Merthiolate" is itself toxic to eggs and the working differential is small. Bacterial counts in low calcium solutions, which prolong the life of the eggs, show no differences which can be correlated with the effects on longevity.

The effect of sodium azide on the respiration of frog muscle. J. N. Stannard.

The results of the present investigation indicate an essential discontinuity in the action of sodium azide on the respiration of resting frog muscle compared with muscle whose oxygen consumption has been forced to higher levels by one of several means.

Under basal, resting conditions (pH 7.38) the respiration is not inhibited by moderate concentrations (M/1000) of azide but instead a stimulation (50–75 *per cent*) occurs. Higher concentrations lead to gradual inhibition after an hour or more. In contrast to the effect on the resting metabolism the increment in respiration caused by the presence of caffeine (0.04 per cent) or isotonic KCl is almost completely eliminated within ten minutes by M/1000 azide. The respiration then remains at the resting level (i.e. the "resting-azide" level) for several hours. The oxidation of para-phenylenediamine is largely, but not completely, inhibited under these conditions, but the increased oxygen consumption due to a carrier such as pyocyanin is not eliminated.

In addition sodium azide inhibits or greatly reduces the burning of carbon monoxide characteristic of resting frog muscle without further effect on the basal oxygen consumption (i.e. the "resting-azide" level persists). In this respect the action of sodium azide differs from that of cyanide, since KCN, over a wide range of concentrations, reduces the resting oxygen consumption and the increment due to carbon monoxide equally.

The effect of azide on frog muscle is, unlike its action on yeast (Keilin), little changed by variations in pH. Even at pH 4.6, although the stimulating action of M/1000 azide does not occur, the rate of inhibition of the resting respiration is only slightly increased. Furthermore, inhibition of the fractions due to caffeine, KCl, etc. takes place readily at pH 7.38.

Respiratory stimulants and gastrulation in Arbacia. A. J. Waterman.

With the purpose of determining the effects, if any, of artificially increased respiratory rate and cleavage retardation on gastrulation in *Arbacia*, the early blastula stage was placed in graded concentrations of 2,4-dinitrophenol, 3,5-dinitro-o-cresol, iodoacetic acid, pyocyanine and methylene blue. The cultures were examined 17–48 hours later and cases of developmental arrest were transferred to fresh sea water.

The results are proportional to the concentration and to the length of exposure. All produce inhibition, retardation and abnormalities in high concentrations and some are possibly very slightly stimulating in weak concentrations. Effective concentrations are different for each reagent. 3,5-dinitro-o-cresol is most toxic and iodoacetic acid least. In sea water inhibited blastulae may form large ectoblastulae (animalization) usually with skeleton; large globular types with skeleton and relatively undifferentiated gut or small endodermal mass; abnormal plutei especially as regards skeleton and arm differentiation, number and size; or typical plutei of small size. There is considerable variation depending upon susceptibility.

The absence of recognizable endoderm does not prevent the formation of skeletal material or the differentiation of ectoderm and even the pluteus body shape. However, the arms never develop. Skeletal modifications include, asymmetry, accessory projections and more extreme modifications. Gastrulation and differentiation are not affected directly by a retarded cleavage rate during the experimental period, nor is gastrulation stimulated. Concentrations strong enough to affect gastrulation either retard or inhibit it. In sublethal concentrations gastrulation may be inhibited for 17–32 hours at least without effect on the capacity to gastrulate in a typical manner. In only a few cases were occasional exogastrulae formed and these did not differentiate. In concentrations of certain of the reagents which possibly stimulated later development, the embryos did not survive as long as the controls.

Does transfer of pronuclei ever occur in conjugation of Paramecium caudatum? Ralph Wichterman.

In an earlier account (*Biol. Bull.*, Oct., 1937) a method was described for studying the activities and sequences of nuclear events during conjugation. A pair of conjugants is isolated and placed in a micro-compression chamber where the organisms are temporarily prevented from spiralling while observations are made with the microscope. Additional information is given here.

Diagrams of conjugation of *Paramecium caudatum* in textbooks show a breakdown of the contiguous membranes shortly after the union of the conjugants. This breakdown of the membranes would lead one strongly to suspect that there is a cytoplasmic transfer. In a study of the living animals such a transfer has not been observed while the conjugants are fused along their oral grooves. Their individual membranes may be seen intact at this stage. Indeed, active cyclosis in each conjugant occurs as it does in the vegetative stage. The micronuclear products as well as the granules and crystals are carried about in the streaming protoplasm.

At the beginning of conjugation, there is a progressive dedifferentiation of the ingestatory structures which appear to pass into a sol state.

After the conjugants separate along the oral groove at the completion of conjugation, a thin clear elastic strand is the last structure to hold the organisms together. This strand, which finally breaks, consists of the stretched and fused apices of the paroral cones.

Calkins and Cull (1907) described chromatin elimination in the second and third "maturation" divisions but stated that it did not occur in the first division. I find it to be very conspicuous in this first division where the long telophasic separation spindle between the daughter nuclei is discarded into the cytoplasm and left to degenerate.

One is struck by the nuclear aberrations especially after the first pre-zygotic division. Also, there is considerable non-synchronous nuclear activity in both

conjugants of a pair, i.e. one conjugant in a pair may show a nuclear stage more advanced than its co-conjugant.

Evidence does not indicate a transfer of pronuclei from one conjugant to another.

More complete data on time relationships with photographs of all the important living stages of conjugation will be presented in the full paper. Motion pictures of all three pre-zygotic divisions are in the process of being made.

Studies in calcification. IV. A contribution to the problem of skeletal calcification in the teleost, Fundulus heteroclitus. E. Alfred Wolf and Alfred S. Levenson.

The Gömöri modification of the silver nitrate method was applied to the study of skeletal calcification in the teleost fish, *Fundulus heteroclitus*, mainly in the adult stage and to a smaller extent in young fish of nine days after hatching. The tissues investigated were: calcified cartilage and membrane bones of the skull, vertebrae, ribs and the so-called "fish bones." In the case of calcified cartilage, the usual staining methods give no safe criterion for the extent of the penetration of calcium salts into the cartilage. The claim that the hypertrophied appearance of the cells in the cartilage is an indicator of those regions where calcium is precipitated was shown not to hold. No relation could be found between the presence of this type of cells in the cartilage and the deposition of calcium as revealed by the silver method. The perichondrial sheath surrounding the cartilage consists of a modified transparent type of fibrous tissue within which calcium is assumed to be deposited. The silver nitrate method applied here gives positive proof of the presence of calcium in this type of tissue. No calcium deposits are present wherever the fibers have not undergone this transformation into the transparent type. Two types of cartilaginous bones were found: in one type the calcium deposits have penetrated from the perichondrium into the cartilaginous parts of the bone; in another type the calcification is confined to the perichondrial sheath. The calcified perichondrium is, in structure, identical with the membrane bones. Vertebrae have a cartilaginous origin, but the cartilage calcifies at a very early stage and can therefore not contribute any essential part to the structure of the fully grown vertebrae of the adult. Ribs maintain their cartilaginous core though they are completely enclosed by the calcified perichondrium.

Mitotic activity of the islands of Langerhans and parathyroids of rats following pituitary extract and colchicine injections.[1] Opal M. Wolf.

The mitotic activity of the endocrine glands of rats following daily injections of an [2] alkaline extract of the anterior lobe of the pituitary gland at 1-cc. and 2-cc. levels has been studied. Control and pituitary-injected animals were etherized the morning following the second, fourth, sixth and tenth days of injection. Nine to fifteen hours before the male animals were etherized 0.3 mg. (per 100 grams of body weight) of [3] colchicine dissolved in distilled water was injected subcutaneously. At autopsy tissues were fixed in Bouins', dehydrated by the anilin oil-dioxan method, cut at 10 micra and stained in Delafield's or Heidenhain's haematoxylin.

By means of an ocular micrometer it was estimated that nine islands of Langerhans in a control rat, 186 grams, averaged 10.5 mitoses per 1,000 cells. The average number of four controls was 7.45. The pituitary-injected rats showed the following counts per 1,000 cells.

[1] The experimental work was performed at Goucher College. I am indebted to Miss Naomi Jaffe for technical assistance. Part of the sections were studied at the Marine Biological Laboratory.

[2] The growth extract was purchased from E. R. Squibb and Sons.

[3] Colchicine U. S. P. was purchased from Merck and Co. in the form of a powder.

	1-cc. Level	2-cc. Level
8 days	15.48	21.26
6 days	—	20.90
10 days	8.89	21.13
	7.45	

Mitoses were counted in five pituitary-injected and three control female rats killed 16 to 23.5 hours after colchicine injection on the fourth day of the experiment.

Hours after Colchicine Injection	Controls	1-cc. Level	2-cc. Level
16.	6.2	23.25	17.28
20.5			27.55
21.		22.81	
21.5		26.12	
23.	4.17		
23.5	5.19		

The average count of the three largest sections of the parathyroid from control male rats showed 2 to 7 cells in mitosis (one control showed 32 cells). The greatest number, 47 mitoses, occurred in a male on the 2-cc. level killed the tenth day of injection. A female killed the fourth day had 57 mitoses on the 1-cc. level for the highest count and another 70 on the 2-cc. level. The highest count for the female controls was 14 mitoses.

Oviducts of pituitary stimulated females, Rana pipiens. Opal M. Wolf.

In an earlier work Wolf (1928) reported that castration caused an involution of the oviducts of female *Rana pipiens* and injections of a water-soluble extract of mammalian ovarian hormone caused an hypertrophy which exceeded the size of the oviducts of normal control animals. It seemed of interest to study the effects of sub-cutaneous injections of bull-frog anterior lobe of the pituitary on the ovary and oviducts of mature female frogs, *Rana pipiens*, during the summer months following the breeding period (having reported, 1937, that isolated adult males in July shed sperm, spermatogenesis occurred, and the thumb-pads on the first finger of the fore limbs became ridged during pituitary injections).

From July 24 to August 17, a total of 15 anterior lobes of the pituitary gland of *Bufo catesbiana* were injected into each adult female *Rana pipiens*, approximately 8.5 cm. in length, tip of mouth to anus. At the end of the period the animals were etherized and the ovaries and oviducts of experimental and control animals were weighed. Tissues were fixed in Bouins' fluid. Histological preparations were made of parts of the ovary and of the oviduct 1.5 cm. from the uterus.

The average weight of the ovaries of injected animals exceeded that of the controls by 62 per cent; the oviducts of the treated animals were 132 per cent more than the controls. Ovulation did not occur but in the experimental animals the nuclei of the larger ova were eccentric or very near the surface. The controls showed centrally placed nuclei. (Moore, 1937, reported that the nuclei of pituitary stimulated ova moved to the surface.) The oviducts of the injected females showed hypertrophied albumen-secreting cells. In relation to the earlier work, it appears that the hypertrophy of the oviducts of the experimental animals is due to an increased output of ovarian hormone from the pituitary-stimulated ovaries.

The effects of Roentgen irradiation on cleavage and early development in the annelid, Chaetopterus pergamentaceus. R. A. Young.

The eggs of *Chaetopterus* were exposed to 7800r per minute for varying lengths of time and under varying conditions in order to compare the direct and indirect effects

of Roentgen irradiation. The results for exposures of several minutes are as follows. Eggs irradiated free in the sea water showed a slight acceleration in the rate of first cleavage, followed by a gradual decrease in the rate of development. Eggs irradiated in the presence of pieces of egg sac showed no significant change in rate of first cleavage, but the later development was slower and slightly abnormal. Eggs irradiated in the egg sacs or in the intact animal showed marked delay in first cleavage and greater abnormalities in larval stages.

The crucial experiment to test the indirect influence of Roentgen irradiation on cells was performed in the following manner. Part of a *Chaetopterus* was shielded during irradiation and eggs were removed from that region immediately after the exposure and inseminated. These eggs showed no radiological effects on their cleavage or later development. But eggs removed from the same region sometime later showed effects quite similar to those shown by eggs taken from the irradiated area. In other words—the effects of irradiation had spread beyond the region directly exposed.

Normally these eggs remain in the germinal vesicle stage while in the egg sacs where the pH is 6.9. The germinal vesicle breaks down after twelve to fifteen minutes in sea water (pH 8.2). Experiments were made to ascertain the influence of the hydrogen ion concentration of the medium surrounding the eggs at the time of irradiation. The results indicate that the more marked response which is shown when eggs are exposed in the egg sacs or in the intact organism is not due to change in pH over this range.

The effect of perisarc removal on regeneration in Tubularia crocea. E. Zwilling.

Several experiments on Tubularia reported by Dr. Barth [1] indicated that a glass capillary tube placed over an end otherwise free to regenerate would inhibit regeneration. In addition, Dr. Barth observed that if the coenosarc at such a free end was retracted into the perisarc, regeneration never occurred. An outgrowth of this work is an experiment in which the perisarc is removed somewhere along the length of the stem and the coenosarc exposed to freely circulating sea water. A number of experiments were performed to determine some of the relationships involved in the regeneration of coenosarc thus exposed. It was found that hydranths do form at the exposed region and that, as a rule, there are two of these and that they are symmetrical mirror-images of each other; that the rate (vol./time) of formation of these hydranths is directly proportional to the amount of coenosarc exposed. In one group of older stems it was found that the morphology of the regenerants varied with the area exposed, namely: with smaller areas small single hydranths formed, with larger exposed areas double hydranths were formed with their distal ends more or less fused. With very large areas two fully-formed hydranths in contact at the oral ends were formed. Laceration of the coenosarc is not responsible for regeneration since exposure of non-lacerated and lacerated coenosarc gave essentially the same results. Further—when the coenosarc is lacerated and the perisarc is replaced no regeneration occurs. It was found that the free ends, especially distal, exert considerable dominance over the exposed region. The greatest number of regenerants was obtained when both ends were ligatured and the exposed region was removed from their influence. An exposed area near the distal end of the stem will completely inhibit the regeneration of a more proximally placed exposed area. It is suggested, in the light of the work of Barth and of Miller (unpublished), that the exposure of the coenosarc tissue to a high oxygen tension is the important factor involved in the stimulation of regeneration.

[1] Barth, L. G., '38, *Physiol. Zoöl.*, **11**: 179.